



Exposure to lipopolysaccharide-type endotoxins and the aging retina

Petra Pei Larsen

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Par / *By* : Petra Pei LARSEN (née FANG)

**EXPOSITION AUX ENDOTOXINES DE TYPE LIPOPOLYSACCHARIDE
ET VIEILLISSEMENT RETINIEN**
**Exposure to lipopolysaccharide-type endotoxins
and the aging retina**

Sous la direction de / *Under the supervision of* : Dr Cécile DELCOURT

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To my family

Exposition aux endotoxines de type lipopolysaccharide et vieillissement rétinien

Les maladies oculaires liées à l'âge, en particulier la dégénérescence maculaire liée à l'âge (DMLA) et le glaucome, sont les principales causes de cécité chez les personnes âgées dans les pays développés. Toutefois, les mécanismes pathologiques sous-jacents de ces maladies multifactorielles ne sont pas encore entièrement compris. Les progrès de la segmentation automatisée des examens en tomographie par cohérence optique (OCT) ont permis de surveiller avec précision les modifications de la structure laminaire de la rétine et du nerf optique *in vivo*, avec une résolution axiale de 5 μm .

Récemment, un rôle potentiel de l'altération du microbiote intestinal dans les maladies oculaires liées à l'âge a été suggéré. Ces altérations du microbiote intestinal peuvent entraîner des concentrations sanguines élevées de pathogènes, d'antigènes et de molécules pro-inflammatoires, y compris les endotoxines de type lipopolysaccharide (LPS).

L'objectif de cette thèse était d'étudier les associations potentielles de l'exposition aux endotoxines de type LPS avec les maladies rétinienues liées à l'âge et les changements morphologiques de la rétine dans la cohorte Aliénor, une cohorte de personnes âgées françaises de la population générale, phénotypées de manière approfondie sur le plan ophtalmologique.

Dans le cadre de cette thèse, nous avons rapporté une association significative des acides gras 3-hydroxylés estérifiés (AG 3-OH) plasmatiques, un proxy de l'exposition à l'endotoxine de type LPS, avec la DMLA précoce dans la cohorte Aliénor. Ceci suggère que l'exposition pro-inflammatoire à long terme aux LPS est impliquée dans les processus physiopathologiques précoces de la DMLA. En outre, nous avons observé une association significative entre les AG 3-OH plasmatiques et l'amincissement de la couche de fibres nerveuses rétinienues péri-papillaires, ce qui indique une implication des LPS dans la neurodégénérescence du nerf optique. Enfin, en utilisant des données longitudinales sur l'épaisseur des couches maculaires, nous avons également pu montrer que les AG 3-OH plasmatiques étaient associés à un amincissement des couches maculaires internes et totales dans l'étude Aliénor.

Résumé

Une autre analyse approfondie de ces données morphologiques longitudinales a révélé que le complexe épithélium pigmentaire rétinien - membrane de Bruch était associé à la DMLA incidente ainsi qu'à la susceptibilité génétique à la DMLA.

Dans l'ensemble, ces observations soulignent l'impact potentiel des LPS et, plus généralement, du microbiote humain sur la santé de la rétine.

Mots clés : Lipopolysaccharide, dégénérescence maculaire liée à l'âge, glaucome, tomographie en cohérence optique

Unité de recherche

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Exposure to lipopolysaccharide-type endotoxins and the aging retina

Age-related eye diseases, in particular age-related macular degeneration (AMD) and glaucoma, are the leading causes of blindness in the elderly in the developed world. However, the underlying pathological mechanisms of these multifactorial diseases are not yet fully understood. The recent progress in retinal imaging, including automated segmentation of optical coherence tomography (OCT) examinations, have allowed to precisely monitor changes of the laminar structure of the retina and the optic nerve with an axial resolution of 5 μm *in vivo*.

Recently, a potential role of altered gut microbiota in age-related eye diseases has been suggested. Such alterations of the gut microbiota may result in elevated blood concentrations of pathogens, antigens and pro-inflammatory molecules including lipopolysaccharide (LPS)-type endotoxins.

The aim of this thesis was to investigate the potential associations of exposure to LPS-type endotoxins with age-related retinal diseases and morphologic changes in the Alienor cohort, a cohort of elderly French community-dwelling people, deeply phenotyped ophthalmologically.

In the frame of this thesis, we reported a significant association between plasma esterified 3-hydroxy (OH)-fatty acids (FAs), a proxy of LPS-type endotoxin exposure, and incident early AMD in the Alienor cohort, suggesting that long term pro-inflammatory exposure to LPS is involved in the early pathophysiological processes of AMD. Further, we also observed a significant association between plasma 3-OH FAs and thinner peripapillary retinal nerve fiber layer, indicating an involvement of LPS in optic nerve neurodegeneration. Lastly, using longitudinal macular layer thickness data, we could also show that plasma 3-OH FAs were linked with thinner baseline inner and total macular layers in the Alienor study.

An additional in-depth analysis of this longitudinal morphologic data revealed that the retinal pigment epithelium – Bruch's Membrane complex was associated with incident AMD and with genetic susceptibility to AMD.

Taken together, these observations highlight the potential impact of LPS and more general of human microbiota in retinal health.

Abstract

Keywords: Lipopolysaccharide, age-related macular degeneration, glaucoma, optical coherence tomography

Research Unit

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- **Larsen PP**, Féart C, Pais de Barros JP, Merle BMJ, Gayraud L, Delyfer MN, Korobelnik JF, Delcourt C. Association of Age-Related Macular Degeneration with a Blood Biomarker of Lipopolysaccharide, a Gut Bacterial Proinflammatory Toxin. *Invest Ophthalmol Vis Sci.* 2023 Nov 1;64(14):47. doi: 10.1167/iovs.64.14.47. PMID: 38032336; PMCID: PMC10691392.
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Table Legend

Table 1. Glaucomatous field defects 32

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Abbreviations

3-OH FAs: 3-hydroxy fatty acids

3C Study: Three-City (Bordeaux, Dijon et Montpellier) Study

AMD: age-related macular degeneration

Alienor Study: Antioxydants Lipides Essentiels Nutrition et maladies OculaiRes Study

AREDS: Age-Related Eye Disease Study

ARMS2: Age-related maculopathy susceptibility 2

BlamD: basal laminar deposits

BlinD: basal Linear Deposits

BM: Bruch's membrane

BMI: body mass index

CFH: complement factor H

CFP: color fundus photograph

CI: confidence Interval

CNV: choroidal neovascularization

E3 Consortium: European Eye Epidemiology Consortium

ELM: external limiting membrane

ETDRS: Early Treatment of Diabetic Retinopathy Study

FDR: false discovery rate

GA: geographic atrophy

GCL: ganglion cell layer

GWAS: genome-wide association study

Abbreviations

HDL: high-density lipoprotein

HTRA1: high-temperature requirement 1

IFN: interferon

IL: interleukin

ILM: internal limiting membrane

INL: inner nuclear layer

IOP: intraocular pressure

IPL: inner plexiform layer

LC-tandem MS: liquid chromatography tandem mass spectrometry

LDL: low-density lipoprotein

LPS: lipopolysaccharide

mRNA: messenger ribonucleic acid

nGA: nascent geographic atrophy

ONH: optic nerve head

ONL: outer nuclear layer

OPL: outer plexiform layer

OR: odds ratio

POAG: primary open angle glaucoma

PRS: polygenic risk score

PSL: photoreceptor segments layer

RNFL: retinal nerve fiber layer

RPD: reticular pseudo-drusen

RPE: retinal pigment epithelium

SD-OCT: spectral domain optical coherence tomography

SDD: subretinal drusenoid deposit

SNP: single nucleotide polymorphism

TNF α : tumor necrosis factor α

TLR: toll-like receptor

VCDR: vertical cup/disc ratio

VEGF: vascular endothelial growth factor

1 Introduction

Due to the demographic change, age-related eye diseases have become increasingly prevalent in the Western population and may lead to visual impairment and blindness. Visual impairment and blindness compromise the ability to perform activities of daily living and can therefore reduce the quality of life of the individual, as well as it is a large socioeconomic burden throughout the world.

In particular age-related macular degeneration (AMD) and glaucoma, are the leading causes of blindness in the elderly in the developed world.^{1,2} However, the underlying pathological mechanisms of these multifactorial diseases are not yet fully understood. Over the last decades, the understanding of eye diseases has greatly increased. Large epidemiological studies have identified environmental risk factors and genetic markers.^{1,3-5} In addition, the advancements of multimodal imaging modalities have allowed to characterize morphologic changes in AMD and glaucoma more distinctly, enabling precise follow-up of disease progression.⁶⁻¹¹ Still, the causes for the development and progress of these diseases remain to be determined.

Recently, a potential role of altered gut microbiota in age-related eye diseases has been suggested. Such alterations of the gut microbiota may result in elevated blood concentrations of pro-inflammatory lipopolysaccharide (LPS)-type endotoxins, which may promote the development and progression of AMD and glaucoma through several mechanisms.

In the present thesis, I analyzed the association of LPS-type endotoxins exposure with age-related eye diseases and morphologic retinal changes in a clinically deeply phenotyped cohort.

Introduction

1.1 Age-related macular degeneration

Age-Related Macular Degeneration (AMD) is a multifactorial and complex disease and the most prevalent cause of blindness in the elderly in industrialized countries.¹ Even though the exact pathogenesis of AMD remains unclear, progressive degenerative processes lead to the accumulation and deposition of undegradable byproducts of the visual cycle in the retina. The affection of the macula restricts central vision, while peripheral vision is often preserved.

1.1.1 Classification and diagnosis

1.1.1.1 Classification

Several different internationally accepted classifications of AMD exist, including the epidemiological Wisconsin classification, the AREDS (Age-Related Eye Disease Study) classification and the Beckman classification.^{12–14} In clinical routine, the Beckman classification is widely used and classifies AMD into three stages according to the severity of fundus lesions (**Figure 1**):

Early AMD rarely causes visual impairment and is described by medium-sized drusen (63–125 μm in diameter). Drusen are clinically visible deposits of abnormal, incompletely degraded material underneath the RPE. They are visualized as yellow spots on color fundus photographs (CFP) and as discrete elevations of the RPE layer using spectral domain optical coherence tomography (SD-OCT).¹⁵ SD-OCT is a non-invasive imaging technique providing cross-sectional images of the retina and has allowed to visualize changes in retinal layers in great detail.¹⁶

Further disease progression to intermediate AMD is associated with larger drusen ($\geq 125 \mu\text{m}$) and/or pigmentary abnormalities of the macula (hypo- and/or hyper-pigmentation). Advanced AMD includes atrophic AMD and neovascular AMD. Atrophic AMD also known as geographic atrophy (GA) is characterized by loss of photoreceptors and RPE cells, which can be seen clinically and on CFP as a sharply demarcated area of depigmentation exposing the underlying choroidal blood vessels.^{1,10,16–18} Using SD-OCT, GA is characterized by a marked atrophy of the outer retinal layers and the RPE. Fundus autofluorescence imaging shows a clearly delineated area of decreased autofluorescence signal intensity in GA and has allowed

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to distinctly characterize different patterns and growth sizes of geographic atrophy.¹⁰

Neovascular AMD corresponds to an invasion of new choroidal blood vessels (choroidal neovascularization, CNV) into the subretinal and/or sub-RPE space that can lead to sight-threatening exudation of fluid and/or blood. CNVs may be visible on fundus examination and CFP as a grayish-green lesion beneath the retina. Fluorescein Angiography is used to visualize the leakage of the CNV. Using SD-OCT, neovascular AMD is characterized by subretinal fluid, subretinal tissue, pigment epithelium detachment and intra-retinal fluid.¹⁶ Both GA and neovascular AMD can coexist in one eye and result in substantial visual decline in most patients.

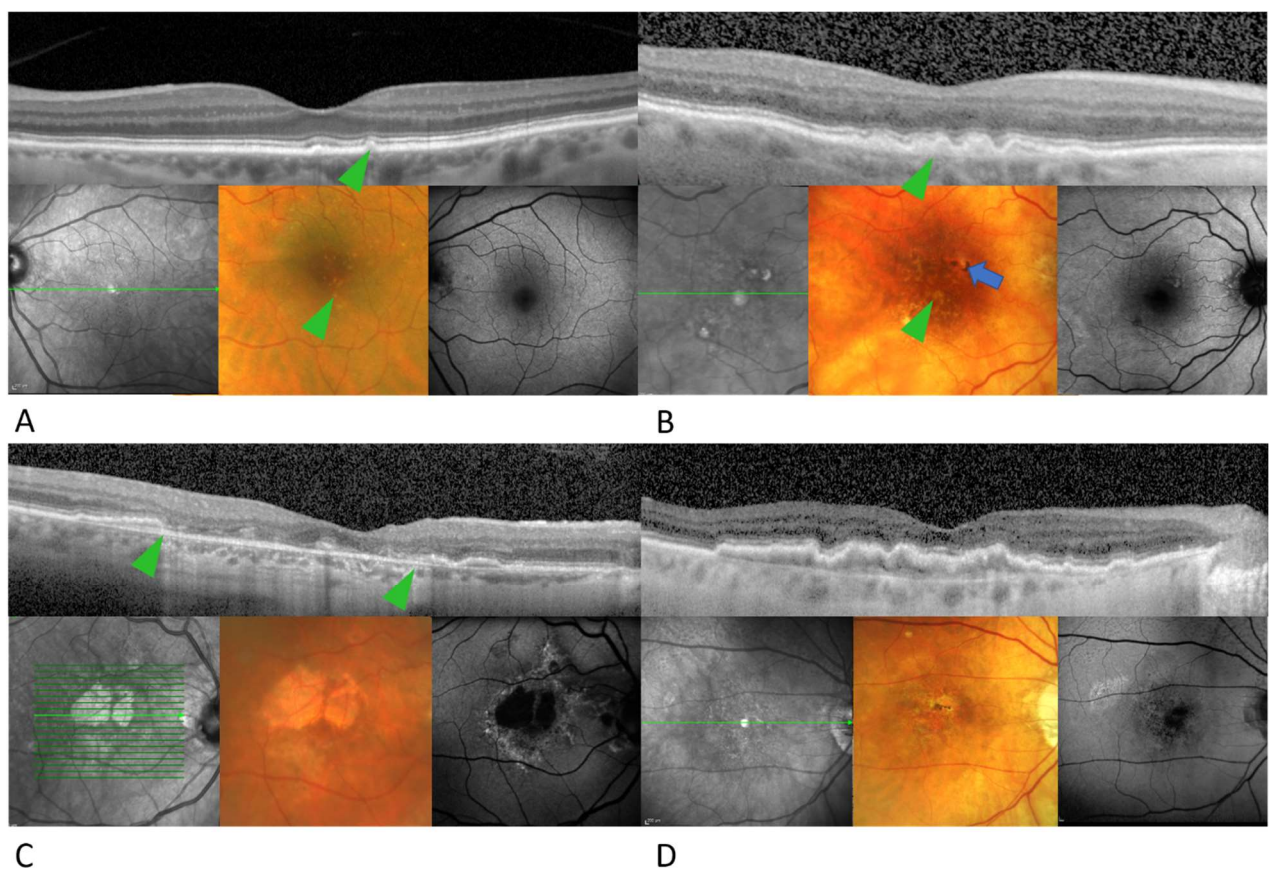


Figure 1. The different stages of age-related macular degeneration (AMD).

Multimodal imaging for each stage of age-related macular degeneration (A-D): top, spectral-domain optical coherence tomography; bottom from left to right, confocal scanning laser ophthalmoscopy near-infrared reflectance, color fundus photography, confocal scanning laser ophthalmoscopy fundus autofluorescence. A. Early AMD: Arrowheads point at a medium-sized drusen (63-125 μm). Drusen are commonly seen as relatively homogenous medium reflective material between the retinal pigment epithelium and the Bruch's Membrane. B. Intermediate AMD: Arrowheads point at a large drusen (≥ 125 μm). Blue arrow points at hyperpigmentation. C. Late atrophic AMD: Arrowhead points at the borders of the atrophic area and D. Late neovascular AMD.

The advent of SD-OCT imaging has strongly impacted the visualization of AMD related changes: the term nascent geographic atrophy (nGA) has been established in 2014 to

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describe changes seen in SD-OCT that predate the development of GA.¹⁷ These changes reflect the underlying atrophy with loss of the photoreceptors and are seen as a subsidence of the outer plexiform layer (OPL) and inner nuclear layer (INL), and/or the development of a hyporeflective, wedge-shaped band within the limits of the OPL.¹⁷ In 2018, the Classification of Atrophy consensus group, proposed to use SD-OCT-based criteria to describe atrophy and introduced the following classification terms¹⁸: complete RPE and outer retinal atrophy (cRORA), incomplete RPE and outer retinal atrophy (iRORA), complete outer retinal atrophy (cORA) and incomplete outer retinal atrophy (iORA). These SD-OCT-based classification terms shall allow for a more detailed description of changes that occur in AMD and thus a higher precision of AMD staging.¹ In 2021, the same group of experts have further reached consensus on 12 multimodal imaging features,⁹ which are associated with GA and risk for GA progression in eyes with AMD.¹⁹ These include retinal layer abnormalities (e.g., ellipsoid zone disruption, hypertransmission of the OCT signal into the choroid or hyporeflective wedges) features frequently seen in eyes with atrophy (e.g., refractile drusen), and features conferring risk for atrophy development (e.g., hyperreflective foci, drusen, and subretinal drusenoid deposits).¹⁹

The latest development in the field of OCT, OCT angiography, allows for non-invasive visualization of blood flow based on motion-contrast.^{20,21} This imaging modality is not part of routine AMD diagnostics, but has made aware of the presence of non-exudative choroidal neovascularization lesions in non-exudative AMD (i.e. early AMD, intermediate AMD or GA).²²

1.1.1.2 Clinical Diagnosis

Clinical diagnosis of AMD involves a combination of patient history, visual acuity testing, dilated fundus examination and multi-modal imaging techniques.

- Patient history may include loss of contrast sensitivity, blurry or distorted central vision, difficulty reading or recognizing faces, and seeing dark or empty areas in the center of their vision (scotomas).
- Visual acuity testing is performed using standard eye chart tests (e.g., a Snellen or Early Treatment of Diabetic Retinopathy Study (ETDRS) chart). A significant drop in

central visual acuity can indicate the presence of AMD. An Amsler grid can provide further information: In AMD, straight lines might appear wavy (metamorphopsias) and some patches may appear blank indicating scotomas.

- After pupil dilation, the retina is examined using an ophthalmoscope or slit lamp. The presence of drusen, pigmentary changes, or abnormalities in the macula is assessed.
- Standard imaging modalities in the context of AMD include CFP and SD-OCT. CFP has been the gold standard for the diagnosis of AMD until the advent of the OCT and also the Beckman classification was based on CFP imaging. CFP captures the natural color of the retina, which helps differentiating retinal lesions based on their color and appearance and remains the method of choice to document hemorrhages and pigmentary changes.⁹ Furthermore, the strength of CFP lies in the wide availability making it useful as large-scale screening tool. SD-OCT has become an integral part of routine AMD clinic and most modern devices have an axial resolution of less than 5 μm . SD-OCT can detect retinal thickening, fluid accumulation, and the integrity of the retinal layers and the RPE.
- In case of suspected CNV, fluorescein angiography is performed. After administration of intravenous fluorescein, the leakage of bloodstream behind the macula can be visualized with a dedicated fundus camera equipped with excitation and barrier filters.²³ This allows for the identification and localization of abnormal vascular processes.
- In the context of GA, fundus autofluorescence imaging allows to clearly delineate the size and pattern of the atrophic area. The non-invasive autofluorescence imaging technique makes use of naturally and pathologically occurring fluorophores of the ocular fundus.^{7,10} Due to the loss of RPE and its inherent fluorophores in GA, the decreased autofluorescence signal correlates with the area of GA, allowing for precise GA segmentation.

1.1.2 Epidemiology

1.1.2.1 Prevalence

A 2014 meta-analysis of pooled global data of people between 45 and 85 years estimated the prevalence of AMD at 8.7 %, affecting 196 million people in 2020 and projected its increase to 288 million people by 2040.²⁴ AMD prevalence increases strongly with age and

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varies greatly by ethnicity, with Europeans carrying the highest disease burden with a prevalence of 12.3 %. Disease prevalence is estimated to be 10.4 % in Hispanics, 7.5 % in Africans and 7.4 % in Asians.²⁴

1.1.2.2 Risk factors

The multifactorial and complex etiology of AMD indicates that genetic, lifestyle and environmental factors contribute to its development. These factors can be broadly classified into two categories: modifiable and non-modifiable.

Modifiable risk factors

Smoking is the most important modifiable risk factor for AMD. It has been shown that current smokers have an approximately three-fold increased risk of advanced age-related macular degeneration, compared with those who have never smoked and that this risk is partly reversible on cessation of smoking.^{25–27}

An association between diet and age-related macular degeneration has been reported in several large epidemiological cohorts, of which especially the association of the Mediterranean diet pattern (characterized by high intake of plant-based food (fruits, vegetables, cereals) and fish and moderate to low intake of red meat and dairy products) with lower risk of late age-related macular degeneration seems of clinical interest.^{28–31}

Several nutrients have also shown associations with AMD, in particular vitamins, carotenoids (lutein and zeaxanthin) and omega-3 fatty acids.^{32–45} Although the literature on dietary supplementation and AMD is rather controversial, the post-hoc analysis of two large randomized controlled trials (AREDS 1 and AREDS 2) including 14,135 eyes have shown that the intake of vitamins A, B6, and C, folate, β -carotene, lutein/ zeaxanthin, magnesium, copper, docosahexaenoic acid and omega-3 fatty acid were significantly associated with decreased risk of advanced AMD. Saturated fatty acid, monounsaturated fatty acid, and oleic acid were significantly associated with increased risk of advanced AMD.⁴⁶ Overall, these results are consistent with the findings regarding the Mediterranean diet, as fruits and vegetables are major sources of carotenoids, vitamins A, C, folate and fish is a major source of omega-3 fatty acids (in particular the long chain docosahexaenoic acid and eicosapentaenoic acid).

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The association between lipids and AMD has been subject of various studies. Through epidemiological as well as Mendelian randomization studies, high-density lipoprotein (HDL)-cholesterol is a well-established risk factor for AMD.^{47–51} By contrast, total cholesterol and triglycerides have inconsistent associations with AMD.^{52,53} The role of HMG-coA reductase inhibitors, also known as “statins”, has also been investigated with mixed results.^{54,55}

In 2021, in a meta-analysis of multiple longitudinal cohort studies by the European Eye Epidemiology (E3) Consortium, physical activity showed a small, but statistically significant, protective effect against the development of age-related macular degeneration.⁵⁶

Other modifiable factors such as cataract surgery, high body mass index, sun exposure, high blood pressure and diabetes have been studied with mixed results.^{27,39,48–51,57–62}

Non-modifiable risk factors

Advanced age is the major risk factor for AMD, with an exponential increase of AMD with age. In populations with European ancestry, a meta-analysis estimated prevalence of advanced AMD to be 1.4 % at 70 years of age, rising to 5.6 % at age 80 and 20 % at age 90.⁶³ No evidence of an interaction between age and sex was reported, indicating no significant sex difference for advanced AMD, although more women are affected by AMD due to their longer life expectancy.^{27,63}

The genetic risk of AMD is considered to be linked to multiple genetic loci of small to modest effect contributing to the disease. However, two loci have been consistently strongly linked to advanced AMD:

- Complement factor H (CFH) Gene, located on chromosome 1, encodes a protein that regulates the complement system, part of the immune system. A specific variant in this gene, known as Y402H (a tyrosine to histidine change at position 402), significantly increases the risk of AMD. This variant affects the protein's ability to regulate inflammation, leading to increased susceptibility to chronic inflammation and damage in the retina.^{64–67}
- Age-related maculopathy susceptibility 2 (ARMS2)/ High-temperature requirement 1 (HTRA1) Locus: located on chromosome 10, the ARMS2 gene and the adjacent HTRA1 gene have been implicated in AMD risk. Variants in this region are associated with

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changes in mitochondrial function and increased inflammation, both contributing to retinal damage.^{68,69} Using the large datasets of a European collaboration study, which included 2068 patients with advanced AMD, the E3 and EYE-RISK Consortia found carriers with the haplotype at ARMS2/HTRA1 to have a particularly high risk of advanced AMD at a relatively early age.⁷⁰

In 2016, a large genome-wide association study (GWAS) identified 52 genetic variants at 34 loci that are significantly associated with AMD. These 52 variants were used to compute a weighted polygenic risk score (PRS). Modelling a population risk score distribution using this PRS was found to explain 27.2 % of AMD variability. This accounts for more than half of the genomic heritability, as heritability of advanced AMD was estimated to be 46.7 % for European-ancestry subjects in the same study. The genes at these 34 loci are primarily involved in three biological pathways: the complement system, lipid metabolism, and extracellular matrix remodeling.³ Using the weighted PRS, the E3 and EYE-RISK Consortia found that the proportion of positive PRS ($PRS > 0$) was 89 % among patients with advanced AMD (1581/1777). This study also confirmed the location of the highest risk loci for advanced AMD to be in ARMS2 (rs3750846) and CHF (rs570618 and rs10922109).⁷¹

Recently, a genome-wide meta-analysis combined the data from the AMD consortium GWAS,^{3,72} the early AMD GWAS,⁷³ the FinnGen R6 AMD GWAS, the Genetic Epidemiology Research on Aging study,⁷⁴ and the United Kingdom Biobank AMD GWAS. Using a PRS built of a total of 65 AMD risk loci, of which nine have not been previously associated with the risk of AMD, resulted into improved prediction accuracy for AMD.⁷⁵

1.1.3 Pathophysiology

AMD is a degenerative disease of the outer retina, affecting most significantly the photoreceptors and RPE.

The exact pathogenesis of AMD is unclear, but is thought to be a progressive multifactorial process. As the strongest risk factor for AMD is age, every hypothesis on the pathogenesis of AMD needs to take into account normal age-related changes of the retina and choroid. Thus, in the following, I will give a short overview of the outer retinal structures, BM and choroid during normal aging and AMD development.

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1.1.3.1 Histopathology of the normal retina, RPE, Bruch's Membrane, and choroid

The retina is an approximately 250 μm thin, neuroectodermally derived tissue. It comprises of the neuroretina, made of nine separate layers (**Figure 2**), and the RPE.⁷⁶ As light enters the eye, it traverses the retinal layers in the following order: i) internal limiting membrane (ILM), ii) retinal nerve fiber layer (RNFL), iii) ganglion cell layer (GCL), iv) inner plexiform layer (IPL), v) inner nuclear layer (INL), vi) outer plexiform layer (OPL), vii) outer nuclear layer (ONL), viii) external limiting membrane (ELM) and ix) photoreceptor segments layer (PSL).

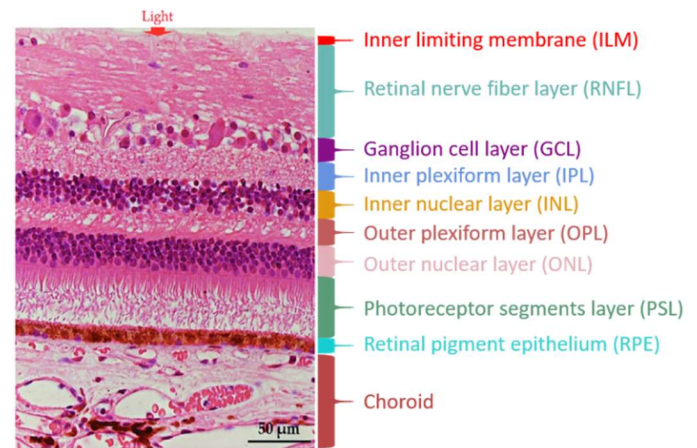


Figure 2. Histological representation of the human retina
(©Image : Triviño A, et al., *Ocular Diseases*, InTech, 2012. doi: 10.5772/48359 under CC BY 3.0 license.)

Photoreceptor cells (i.e., rods and cones) are sensory cells that convert photons of light into electrical impulses, thereby initiating signal transduction to the brain. Their cell nuclei are located in the ONL extending their axonal terminals into the OPL, whereas their inner and outer segments are found in the PSL. The outer segment includes the photoreceptor discs, which contain photopigment, which reacts to light; the inner segment includes the structures responsible for intracellular metabolism and transport.⁷⁷

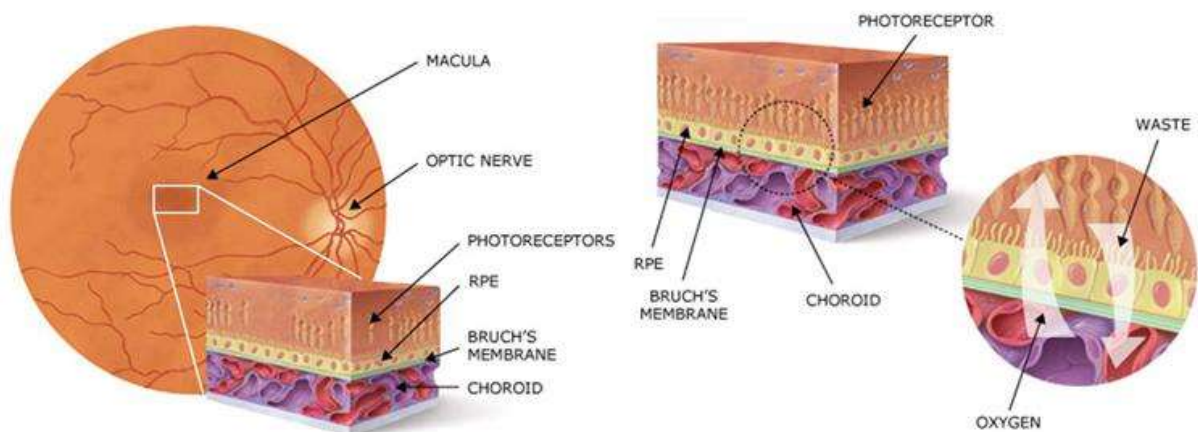


Figure 3. The retinal pigment epithelium (RPE).

The RPE consists of a cellular monolayer located directly beneath the neurosensory retina and above the choroid, and plays a key role in the pathogenesis of AMD. (©Image: AMD Alliance under CC0)

The RPE is a cell monolayer located between the neurosensory retina and the BM and plays a key role in the pathogenesis of AMD (**Figure 3**). The RPE serves a variety of metabolic and supportive functions essential for function and survival of the retinal photoreceptors,

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including the maintenance of the blood-retina barrier, participation in the visual cycle and phagocytic uptake and degradation of photoreceptor outer segments.⁷⁸

The BM lies between the choriocapillaris and the RPE. It is not a membrane in the traditional sense, but represents interstitial connective tissue, which can be regarded as both the innermost aspect of the choriocapillaris and the outermost aspect of the retina. Electron microscopy revealed a pentalaminar structure consisting of i) the basal lamina of the RPE, ii) the inner collagenous layer, iii) the elastic layer, iv) the outer collagenous layer and the basal lamina of the choriocapillaris.^{76,79}

The choroid is a vascular bed between BM and the sclera. The structure of the choroid into the following layers: i) the choriocapillaris, a single layer of fenestrated capillaries that supplies blood to the RPE and outer neurosensory retina, ii) the Sattler's Layer, consisting of intermediate arterioles and venules and connecting the choriocapillaris to iii) the Haller's Layer, the outer choroidal layer containing the large choroidal vessels.⁷⁹

1.1.3.2 Changes of the outer retina during normal aging and AMD development

Changes of the outer retina – BM- choroid complex in normal aging

In normal aging, the outer retina undergoes a number of anatomical and physiological changes including:

- i) Thickening of the BM: The five layers of BM become less sharply demarcated with age and a linear relationship between BM thickening and age has been demonstrated (from 2 μm in < 10 years old to 4.7 μm in > 80 years old).⁸⁰
- ii) Loss of Photoreceptors: This predominately affects rods.⁷⁷
- iii) Choroidal Thinning: Histopathological evidence indicates that loss of choriocapillaris is the most pronounced effect of ageing in the outer retina –BM - choroid complex,^{81,82} and reduction in choriocapillaris blood flow may increase risk for AMD and CNV.⁸³
- iv) RPE Changes: Those changes include accumulation of lipofuscin (byproducts of photoreceptor outer segment turnover), reduced number of mitochondria, loss of cristae and matrix density, decrease in melanosomes, and increase in toxic visual cycle byproducts.^{84–87}

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- v) Development of hard drusen: Drusen are acellular, lipid-rich incompletely degraded metabolic products and can be categorized by their size, shape, and location. Hard drusen are $< 63 \mu\text{m}$, have sharp borders and accumulate as part of the normal retinal aging process.

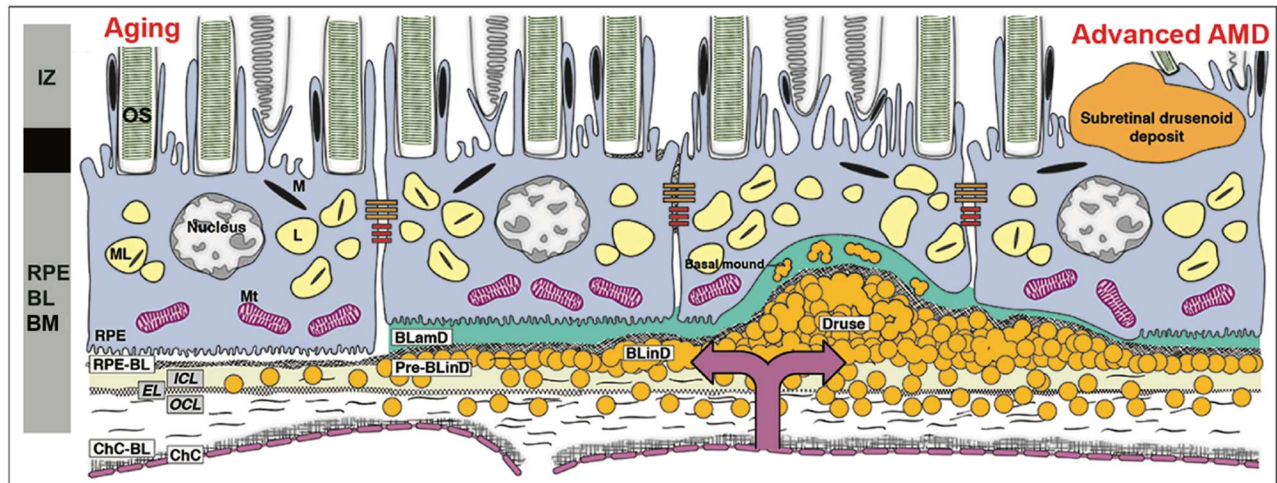


Figure 4. Changes in the RPE-BM complex in aging and age-related macular degeneration.

Between the basal lamina of the RPE (RPE-BL) and the inner collagenous layer (ICL) of Bruch's membrane accumulate drusen and in some eyes type 1 macular neovascularization (MNV, double headed arrow). Soft druse material is found also in basal linear deposits (BLinD) and in basal mounds within basal laminar deposits (BLamD). BLinD may be continuous with, yet distinct from drusen, which are dome-shaped. The schematic bar at the left shows the third and fourth outer retinal reflective bands of optical coherence tomography. The third band, interdigitation zone (IZ), corresponds to interleaved photoreceptor outer segments and RPE apical processes. Components of the fourth band include the RPE – BM complex. BM, Bruch's Membrane; BLamD, basal laminar deposit; BLinD, basal linear deposit; ChC, choriocapillaris; ChC-BL, ChC basal lamina; EL, elastic layer; ICL, inner collagenous layer; IZ, interdigitation zone; M, melanosome; ML, melanolipofuscin; MNV, macular neovascularization; Mt, mitochondria; OCL, outer collagenous layer; OS, outer segments of photoreceptors; RPE, retinal pigment epithelium; RPE-BL, RPE basal lamina.

(©Image: Sura AA, et al., Invest. Ophthalmol Vis Sci, 2020;61(13):19.⁸⁸ doi:10.1167/iovs.61.13.19 under CC BY-NC 4.0 license)

Changes of the outer retina–BM–choroid complex in AMD

To the current state of research, the transition from normal ageing processes to AMD does not begin at a precise "moment". AMD typically develops on the grounds of normal age-related changes gradually over time (**Figure 4**). The clinical diagnosis of AMD depends on observable changes in the retina, with the presence and size of drusen used to define the disease. In AMD, loss of normal BM architecture as well as BM thickening greater than with aging alone is observed. Further, cumulative oxidative damage, immune dysregulation and chronic inflammation together with accumulation of lipofuscin is thought to induce cell injury and dysregulate RPE function.⁸⁹ The RPE mosaic, a normally uniform hexagonal array,

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begins exhibiting pleomorphism.^{90,91} As RPE dysfunction increases, various deposits, explaining partly the BM thickening, are observed⁹²:

- i) Basal laminar deposits (BlamD) are extracellular debris consisting of basement membrane proteins and long-spacing collagen and are histologically found between the RPE plasma membrane and its basal lamina and may indicate a strong risk for AMD progression.^{88,93} Localized detachments of the BlamD may result in the formation of soft drusen.⁷⁶
- ii) Basal Linear Deposits (BlinD) are located external to the RPE basement membrane. They consist of lipid-rich vesicular material similar to soft drusen and are usually located in the inner collagenous zone of BM. BlinD may represent progression of BlamD, are found in association with soft drusen and RPE detachments and their amount is an indicator of the degree of RPE and photoreceptor degeneration.^{91,92}
- iii) Soft drusen are localized deposits between the RPE basement membrane and BM and may represent localized accumulations of BlinD.^{94,95} They are by definition larger than hard drusen (medium sized soft drusen: 63-125 μm and large sized soft drusen: $\geq 125 \mu\text{m}$). By activating complement deposition in BM, soft drusen play a role in increased vascular endothelial growth factor (VEGF) production by RPE cells.⁹⁶ Large, soft, bilateral, and numerous drusen together with RPE pigmentary changes in the macula are a significant risk factor for the progression to advanced AMD.⁹⁷
- iv) Reticular pseudo-drusen (RPD) or subretinal drusenoid deposit (SDD) consist of an accumulation of extracellular debris above the RPE. The term RPD was used to describe “yellow interlacing network 125–250 μm wide, appearing first in the superior outer macula and extending circumferentially and beyond”.⁹⁸ With the advent of SD-OCT, they were also named SDD as they appeared as hyper-reflective deposits internal to the RPE on OCT.⁹⁹ SDD have been associated with a more than four-fold risk of 5-year progression to advanced AMD compared to eyes with only drusen.^{100,101}

Analysis of the protein and lipid components on drusen composition revealed a large number of immune fragments within drusen including complement fragments.¹⁰²

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Complement system activation is thought to be strongly linked to the pathogenesis of drusen and the evolution of pathological changes in AMD, including choriocapillaris degeneration.¹⁰³ Also, many genetic alleles (e.g. CFH, complement factors B, C2 and C3, CFI, ARMS2, etc.) that have been reported to increase the risk for AMD are implicated in the immune system.¹⁰⁴

Further disease progression is associated with increasing RPE dysfunction and eventually degeneration of the RPE and thus death of the corresponding photoreceptor cells. However, photoreceptor loss (in particular rods), has been observed to occur prior to RPE dysfunction.¹⁰⁵ Eventually, in susceptible individuals, RPE dysfunction exacerbates rod loss and cone loss begins, leading to geographic atrophy.⁷⁶

It has also been hypothesized that AMD may develop on the ground of a primary microangiopathy that accelerates further pathological events by reducing blood supply to the photoreceptors and the RPE, and by diminishing the lipoprotein clearance from the RPE and BM.¹⁰⁶ The pro-angiogenic properties of oxidized lipoproteins may further lead to microglia activation and release of cytokines, which promote VEGF expression in RPE cells.¹⁰⁷ Further pro-inflammatory crosstalk coupled with oxidative stress presumably leads to loss of the RPE barrier integrity and permits secreted VEGF entry into the retina, which eventually leads to abnormal growth of blood vessels known as CNV.^{1,81,108}

1.1.4 Treatment

In 2007, intravitreal injections of VEGF inhibitors were approved for the therapy of neovascular AMD.^{109,110} This marks a milestone in the treatment of AMD, as for the first time, it allowed for a significant improvement of visual function in AMD patients.¹¹¹ However, VEGF inhibitors are not curative, often necessitating life-long injections, and cannot prevent a slow deterioration in function in treated neovascular AMD in most cases.¹¹² For the treatment of GA, pegcetacoplan, a complement C3 inhibitor, was approved in 2023 in the USA, following the observation of reduced GA growth compared with sham treatment.^{113,114} However, to date, no effective therapies for early or intermediate AMD exist and none of the approved therapies for advanced AMD are curative.

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1.2 Glaucoma

Glaucoma is the worldwide leading cause of irreversible vision loss, comprising a heterogeneous group of diseases characterized by irreversible progressive loss of retinal ganglion cells.^{115,116} All forms of glaucoma are characterized by loss of retinal ganglion cells, thinning of the retinal nerve fiber layer, and cupping of the optic disc.

1.2.1 Classification and diagnosis

1.2.1.1 Classification

According to the anatomy of the anterior chamber angle, glaucoma is divided into open-angle glaucoma and angle-closure glaucoma (**Figure 5**). Glaucoma is further classified into primary or secondary, where primary cannot be contributed to any other known cause and secondary glaucoma develops as a complication of injury or another medical condition, such as pigment dispersion syndrome, pseudoexfoliation or uveitis. Several other forms of glaucoma exist, including congenital glaucoma.

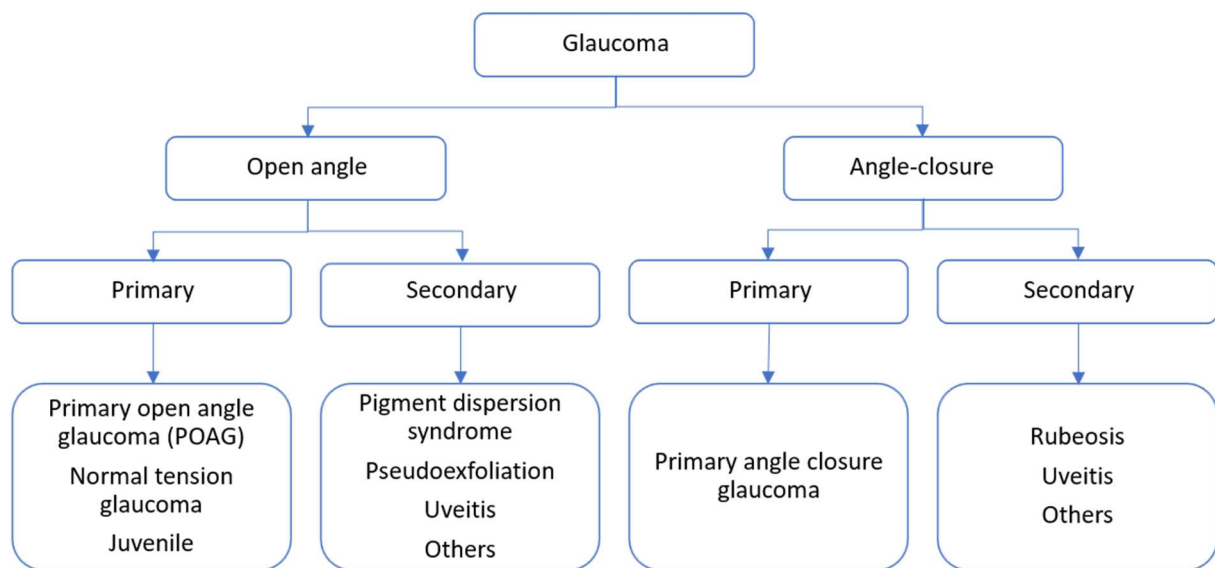


Figure 5. Classification of glaucoma by anatomy

In addition, in epidemiological research, the term glaucoma is applied only for individuals with confirmed, visually significant damage to the optic nerve according to the classification by Foster et al.¹¹⁷ Following this definition, glaucoma is understood as an optic neuropathy characterized by i) distinctive structural damage to the optic nerve and ii) related functional impairment, which can result from a range of underlying pathological processes.

i) Structural damage

Glaucomatous optic neuropathy presents with a characteristic pattern of damage to the optic nerve head, including pallor of the optic disc and a more pronounced cupping at the superior and inferior poles of the optic disc. Due to the vertical elongation in glaucoma-related damage, the vertical cup/disc ratio (VCDR, **Figure 6**) has been established as a relatively robust indicator of glaucomatous loss of the

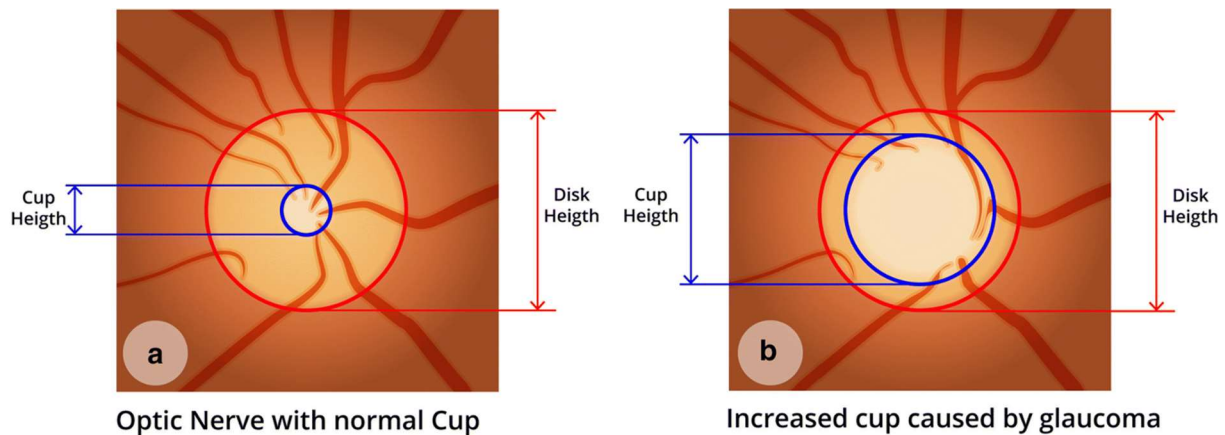


Figure 6. Schematic figure of the vertical cup/disc ratio (VCDR)

A. Optic nerve with normal VCDR and B. with increased VCDR (@Image: Barros D, et al., Biomed Eng Online, 2020;19(1):1-22.⁹⁶ doi:10.1186/s12938-020-00767-2 under CC BY-NC 4.0 license)

neuroretinal rim. A normal VCDR typically ranges from 0.3 to 0.4, meaning the cup is about 30-40 % the size of the disc. A higher ratio (e.g., 0.6 or higher) may suggest glaucomatous damage, as it indicates that the cup is larger than normal. However, the choice where “glaucomatous” starts is arbitrary due to overlap between the range of VCDRs in those with and without glaucomatous visual loss. Foster et al. propose using a statistical convention where a probability of less than 2.5 % indicates a significant deviation from normal.¹¹⁷ In most populations, this corresponds to a VCDR of 0.65 to 0.7. Other indicators of glaucomatous optic nerve damage are a minimal rim/disc ratio less than 0.1 and an asymmetry of the VCDR between the 2 eyes of 0.2 or more.^{117–119}

ii) Functional impairment

In glaucoma, damage to the optic nerve often result in specific patterns of vision loss and generally share certain characteristics, which are outlined in **Table 1**.

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Table 1. Glaucomatous field defects

Characteristics of glaucomatous field defects ¹¹⁷
• Asymmetrical across the horizontal midline (in early/moderate cases) • Located in the mid-periphery (in early/moderate cases) • Clustered in neighboring test points • Reproducible on at least two occasions • Not explained by any other disease • Considered a valid representation of the subject's functional status (based on performance indices such as false positive rate)
Classical scotomas in glaucoma (ordered from generally early to late defects) ^{120,121}
• Paracentral Scotomas: Small, localized areas of vision loss within Bjerrum's area (Figure 7), which may also be associated with nasal steps. • Nasal Step: A sharp visual field loss near the nasal side of the visual field. • Peripheral field defects: Peripheral defects may occur in early or late stages of glaucoma. • Seidel scotoma: Paracentral sickle-shaped scotoma that is a superior or inferior extension of the blind spot. • Bjerrum or arcuate scotoma: Arc-shaped scotoma that connects with the blind spot, following the nerve fiber layer pattern, either in the upper or lower visual field to reach the horizontal line. Peripheral breakthrough may occur due to damage of nerve fibers. • Ring or bi-arcuate scotoma: Arcuate scotomas in the upper and lower visual field join to form a ring. • Roenne's central nasal step: It is created when two arcuate scotomas do not proceed at the same rate in the upper and lower portions of the eye. Consequently, a step-like defect is created where the arcuate defects meet at the median. • Tunnel or tubular vision: Since macular fibers are most resistant to glaucomatous damage, loss of peripheral vision may occur in late stage glaucoma, leaving only a small constricted central visual field intact.

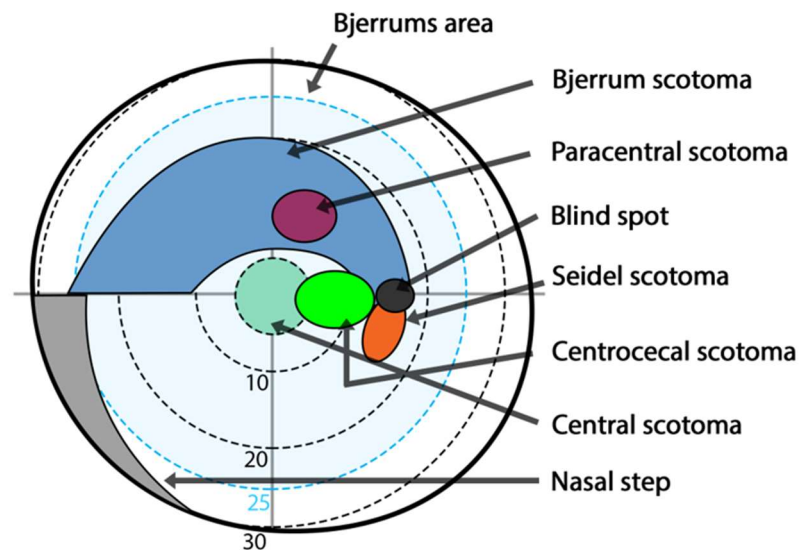


Figure 7. Classical scotomas in glaucoma.

Bjerrum's area corresponds to the central 25° of vision. (©Image: Mattimussi under CC BY-SA 4.0 license)

However, additionally to the patterns listed in **Table 1** and illustrated in **Figure 7**, also diffuse damage to the visual field is observed in glaucoma. Thus in epidemiological studies, the glaucoma hemifield test graded “outside normal limits” and a cluster of three contiguous points at the 5 % level on the pattern deviation plot, using the threshold test strategy with the 24-2 test pattern of the Zeiss-Humphrey field analyzer has been established as glaucomatous visual field loss.¹¹⁷

Of note, the cohort studied in this doctoral thesis, which will be presented in detail in the methodology section, consists of French elderly individuals. As the predominant type of glaucoma in this cohort is the primary open angle glaucoma (POAG), the following paragraphs will focus on POAG.

1.2.1.2 Diagnosis

Early in the disease, POAG is often asymptomatic because peripheral vision is affected first. Central vision remains intact until the later stages, making early detection challenging and diagnosis is frequently delayed.^{122,123} As disease progresses, the optic disc often undergoes excavation due to the loss of neural tissue. This cupping is a hallmark of POAG and is often used as a diagnostic sign during clinical evaluations.¹²⁴

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Clinical diagnosis of POAG involves a combination of patient history, visual acuity testing, tonometry, gonioscopy, optic nerve head evaluation, perimetry, pachymetry and retinal nerve fiber measurement techniques.

- Patient history may be asymptomatic in early-stage POAG. Patients with more advanced disease may report peripheral vision loss, difficulty adjusting to low light, or seeing halos around lights.
- Visual acuity testing is performed using standard eye chart tests (e.g., a Snellen or ETDRS chart). No drop in central visual acuity is expected until advanced POAG.
- Goldmann applanation tonometry is the gold standard for measuring intraocular pressure (IOP) and indirectly measures the IOP by assessing the force needed to flatten a predetermined surface area of the cornea. Often non-contact tonometry is performed as a screening method using a burst of air to flatten the cornea and measure IOP without direct contact.
- Gonioscopy involves the application of a special lens with mirrors placed on the cornea to examine the angle where the iris meets the cornea. The commonly used clinical grading system of Shaffer describes the degree to which the angle is open.¹²⁵
- Optic nerve head evaluation includes the examination of the optic nerve head for signs of damage, such as increased cupping, thinning of the neuroretinal rim, or asymmetry between the two eyes. High-resolution color photographs (**Figure 8**) of the optic nerve are taken for documentation and to monitor changes over time, whereby this is gradually being replaced by other imaging technologies.

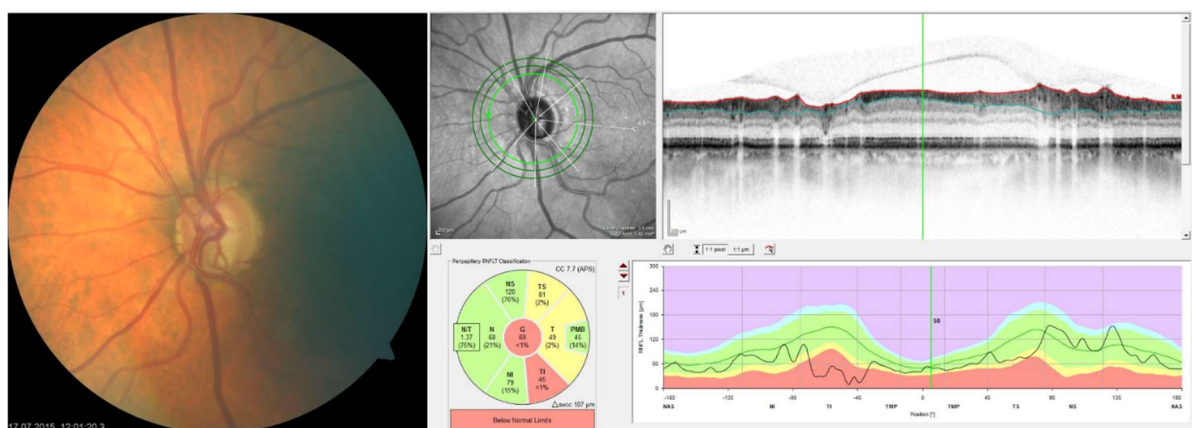


Figure 8. Color fundus image and RNFL measurement of a patient with glaucoma. Example of color fundus image (left) showing increased vertical cup/disc ratio and RNFL examination using SD-OCT (right) showing RNFL loss of a patient with glaucoma.

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- Perimetry or visual field testing helps to detect and quantify visual field defects, which are a hallmark of POAG.
- Pachymetry measures the thickness of the cornea, as thinner cornea can lead to underestimation of IOP, while a thicker cornea may lead to overestimation.
- Retinal nerve fiber measurement is nowadays widely performed using SD-OCT (**Figure 8**). As already described in section 1.1.1, SD-OCT is a non-invasive imaging technique providing cross-sectional images allowing to characterize changes in each retinal layer in great detail. Indeed, the noninvasive SD-OCT-technology provides fast high-resolution imaging of the optic nerve, providing reproducible measurements of peripapillary RNFL thickness with an axial resolution of 5 μm .^{20,126} The quantification of the RNFL enables the identification of patients at risk and at early stage of disease as well as the follow-up of RNFL thinning in glaucoma progression.⁸

1.2.2 Epidemiology

1.2.2.1 Prevalence

Data from population-based surveys indicate that globally more than 3.5 % of adults aged 40 to 80 years are affected by glaucoma, which equates to more than 95 million people.^{2,116} Being the leading cause of irreversible blindness worldwide, it was estimated that 3.6 million people were blind due to glaucoma among adults aged 50 years and older in 2020.¹²⁷

The estimated global prevalence of primary open-angle glaucoma was 3.1 % and that of primary angle-closure glaucoma was about 0.5 %. In people with European, Hispanic and African ancestry, the most common form of glaucoma is primary open angle glaucoma, with the highest prevalence of primary open-angle glaucoma in people with African ancestry (4.2 %). Primary angle closure glaucoma is more common in Asia, with a prevalence of 1.1 %.¹¹⁶

Men were more likely to be diagnosed with primary open-angle glaucoma than women (odds ratio [OR] 1.36).¹¹⁶

In a clinical context, it is important not to overlook the significant proportion of undiagnosed cases of glaucoma. A major systematic review and meta-analysis from 2021, analyzing

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population-based studies on the global extent of undetected glaucoma, found that over half of all glaucoma cases went undiagnosed across various geographical regions.¹²³

1.2.2.2 Risk factors

Risk factors for glaucoma can be categorized broadly at the individual level or the ocular level.¹¹⁵ The most important risk factors for glaucoma (both open-angle glaucoma and angle closure glaucoma) are age, elevated intraocular pressure, and positive family history for glaucoma.¹²⁸ For open-angle glaucoma, myopia has been also consistently reported as a risk factor.²

Mutations in several genes have been associated with various forms of glaucoma. However, being a complex polygenic disease, only less than 5 % of glaucoma have reported to be linked to rare single mutations. Polymorphisms in over 125 genes have been reported to be associated with glaucoma and at least 112 genomic loci with elevated IOP.¹²⁹ Myocilin (MYOC) mutations have been reported in 2-4 % of early-onset primary open-angle glaucoma and optineurin (OPTN) as well as TANK binding kinase 1 (TBK1) in normal-tension glaucoma. Other affected genes are involved IOP regulation, neuroprotection, or other processes critical to optic nerve health.² Recently, a multitrait analysis of a large-scale GWAS proposed a more comprehensive glaucoma PRS based on 2673 independent SNPs associated with glaucoma.¹³⁰ The study reports that this new PRS improves the ability to predict both disease susceptibility and progression. This would enable more personalized risk assessments, and thus potentially earlier detection and intervention in individuals at higher risk for developing glaucoma. To account for inter-ancestral differences in genetic susceptibility, a largescale, multi-ancestry meta-analysis calculated a PRS based on 127 POAG-associated variants and provided cross-ancestry and ancestry-specific effect estimates for individuals of European and African ancestry. The PRS was significantly associated with POAG in both groups; however, a higher proportion of individuals with European ancestry, compared with African ancestry, were consistently categorized in the top PRS decile.¹³¹

Other associations have been reported, but still need to be confirmed, in particular the associations with smoking,^{132–136} nutrition (in particular the Mediterranean-DASH Intervention for Neurodegenerative Delay (MIND) diet,¹³⁷ omega 3 fatty acids,^{135,138}

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nicotinamide (vitamin B3), folate (vitamin B9) and other vitamins,^{139–143} salt intake,¹⁴⁴ alcohol and coffee consumption^{145–148} and use of systemic calcium channel blockers.^{149,150} In addition, recent studies have evidenced an increased risk for glaucoma and RNFL thinning in individuals exposed to high levels of air pollution, in particular fine particles (with diameter inferior to 2.5 μm).^{151–155}

1.2.3 Pathophysiology

The multifactorial nature of the pathophysiology of POAG so far prevents the modelling of this disease *in vitro* and *in vivo* and thus the exact pathogenesis remains unclear. However, it is known that the interaction between IOP, vascular dysregulation, neuroinflammation and oxidative stress are at play, eventually leading to optic nerve injury.^{115,156}

As glaucomatous optic neuropathy presents with a characteristic pattern of damage to the optic nerve head, I will first give a short overview on the healthy optic nerve head and then describe the changes caused by glaucoma.

1.2.3.1 The optic nerve head

The optic nerve head (ONH, **Figure 9**) is located at the posterior pole of the eye and is the point of exit for ganglion cell axons leaving the eye. In emmetropic eyes, the ONH is located 4 to 5 mm to the nasal and superior side of the fovea.¹⁵⁷ As there are no photoreceptors at the ONH, it corresponds to the blind spot in the visual field of each eye. The ONH represents the beginning of the optic nerve and carries approximately 1–1.2 million afferent nerve fibers from the eye toward the brain. The ONH is also the entry point for the major arteries that supply the retina with blood, and the exit point for the veins from the retina.

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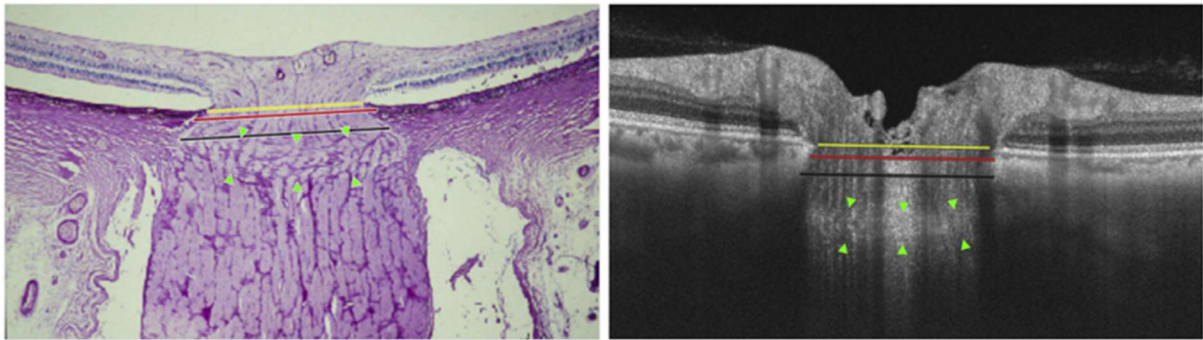


Figure 9. The optic nerve head

Histologic image of the optic disc (left) and SD-OCT scan (right) showing the three layers of the optic nerve head canal, including Bruch's membrane opening (yellow line), the choroidal opening (red line), and the opening of the peripapillary scleral flange (black line), covered by the lamina cribrosa (green triangle), shown in a healthy optic nerve.

(©Image: Wang YX, et al., *Prog Retin Eye Res.* 2021 Jul;83:100933.¹⁵⁸ doi: 10.1016/j.preteyeres.2020.100933 under CC BY-NC 4.0 license)

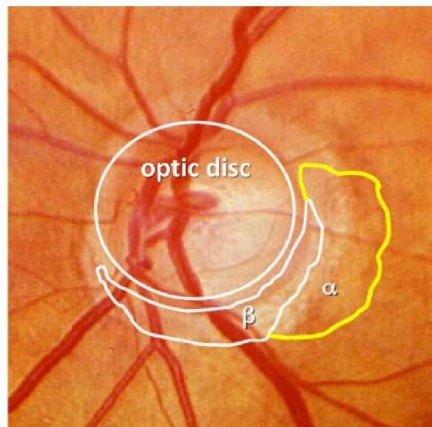


Figure 10. The optic disc and the parapapillary atrophy

The optic nerve head can morphologically be differentiated into the optic disc with the lamina cribrosa as its basis, and the parapapillary region with zones alpha and beta. The alpha zone is characterized by the presence of BM and RPE, while the beta zone is characterized by the presence of BM and the absence of RPE. (©Image: Sun J, et al., *Int J Biomed Imaging.* 2015;2015:528626. doi: 10.1155/2015/528626 under CC BY license)

Histologically, the ONH is composed of three layers: the inner layer formed by the opening in Bruch's membrane (BM), the middle layer by the opening in the choroid, and the outer layer by the opening in the peripapillary scleral flange.¹⁵⁸ The outer layer is covered by the lamina cribrosa, which permits the passage of retinal ganglion cell axons and blood vessels while largely preventing fluid exchange between the vitreous cavity on the inside and the optic nerve and retrobulbar cerebrospinal fluid space on the outside. Morphologically, the ONH is defined by the optic disc, which includes the region with the lamina cribrosa at its basis, and the surrounding peripapillary area, which includes the parapapillary zones alpha and beta.^{159,160} The more peripheral alpha zone is understood to bear no pathological value and is

present in almost all eyes.¹⁶¹ It is characterized by the presence of BM and RPE and shows irregularities due to pigmentary variation in the parapapillary RPE. The beta zone is defined by the presence of BM while RPE is absent (**Figure 10**).¹⁵⁹ The baseline presence and the size of parapapillary atrophy in the beta zone has been correlated to glaucomatous visual field and optic nerve damage in several large prospective and retrospective studies.^{162–164} In the

Introduction

literature, also further parapapillary zones (gamma and delta) are discussed, which are considered to be characteristic for myopia.¹⁵⁸

1.2.3.2 Changes leading to glaucomatous optic nerve head damage

There are two main theories leading to optic nerve injury:

- i) The mechanical theory: To maintain intraocular pressure, the ciliary body of the human eye continuously produces aqueous humor. This fluid flows through the pupil, and drains through the trabecular meshwork into Schlemm's canal. In POAG, there is an imbalance between the production and drainage due to increased resistance to aqueous outflow through the trabecular meshwork, leading to elevated IOP. Elevated IOP is a major risk factor for glaucoma as it causes mechanical stress on the optic nerve head, leading to its gradual damage including visible cupping due to the loss of neural tissue and eventually, axonal loss and RGC death. However, while elevated IOP is a significant risk factor, POAG can also occur with normal or low IOP (normal-tension glaucoma). This indicates the presence of other mechanisms that contribute to optic nerve damage.
- ii) The vascular theory: Particularly in normal-tension glaucoma, there is a primary vascular dysregulation where blood flow to the optic nerve is not adequately regulated. There may be an abnormally low cerebrospinal fluid pressure in the optic nerve subarachnoid space resulting in a large pressure gradient across the lamina.^{165,166} This can lead to chronic ischemia and hypoxia, leading to the release of toxic substances, including reactive oxygen species (ROS), that damage retinal ganglion cells and their axons. Elevated IOP can also lead to secondary vascular changes, including compression of blood vessels within the optic nerve head, further reducing blood supply and contributing to optic nerve damage.¹⁵⁶

The loss of RGCs in POAG occurs primarily through apoptosis, a programmed cell death process. Elevated IOP, oxidative stress and neuroinflammation can trigger apoptosis by activating signaling pathways that lead to cell death, contributing to the progressive loss of vision. The retina is particularly vulnerable to oxidative damage due to its high metabolic

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activity. Microglial cells, the resident immune cells in the retina, become activated in POAG, releasing pro-inflammatory cytokines and neurotoxic substances that further damage RGCs.

1.2.4 Treatment

Although to date many different types of medication and surgery exist for POAG, their common aim is to prevent further damage as damage to the optic nerve remains irreversible.

All treatment options primarily aim to lower and control IOP in a target range to prevent or slow down optic nerve damage in order to preserve visual function and quality of life.¹⁶⁷

Treatment strategies include medications, laser therapy, and surgical interventions. The choice of treatment depends on the type and severity of glaucoma, as well as the patient's overall health and response to therapy.

Medications are usually the first line of treatment for glaucoma, and they work by either reducing the production of aqueous humor or increasing its outflow. These are administered as eye drops or, less commonly, oral medications. Laser treatments are commonly used when medications are not sufficient to control IOP or as an initial treatment in certain cases. Surgery is considered when IOP cannot be controlled with medications or laser treatments alone, or when the patient has advanced glaucoma.

Since the large number of available treatment options is beyond the scope of this thesis, I would like to refer the interested reader to the comprehensive discussion of existing glaucoma treatment in “the Primary Open-Angle Glaucoma Preferred Practice Pattern” of the American Academy of Ophthalmology.¹⁶⁷

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1.3 Lipopolysaccharide (LPS)-type endotoxins

Although many recent studies have enhanced our understanding of the role of risk factors and molecular mechanisms involved in age-related eye diseases, the multifactorial nature of AMD and glaucoma so far prevents the modelling of these disease *in vitro* and/or *in vivo*. However, as described in the sections above, inflammatory processes have been identified in both diseases.

LPS-type endotoxins are components of the Gram-negative bacteria. They were initially termed 'endotoxins' to differentiate them from 'exotoxins,' as they were thought to be toxins within the bacteria, while exotoxins were released from the bacteria. However, it is now understood that endotoxins are also released by bacteria and that their toxicity arises from the host's excessive inflammatory response to them.¹⁶⁸ As component of the outer membrane of Gram-negative bacteria, LPS is naturally found in the human microbiota and recognized as trigger of inflammation. Its immunostimulatory effects include an altered production of key inflammatory mediators, such as cytokines and chemokines.

1.3.1 The structure and main signaling pathway of LPS

LPS is a large glycolipid consisting of three structural domains: a core oligosaccharide, an O-antigen polysaccharide and Lipid A (**Figure 11**).^{169,170} Lipid A is composed of an acylated β -1'-6-linked glucosamine disaccharide that forms the outer leaflet of the outer membrane of Gram-negative bacteria. Two molecules of 3-hydroxy (3-OH) fatty acids (FAs) are esterified to the glucosamine backbone of Lipid A, and two are attached in amide linkage.¹⁷¹ Lipid A acts as the pathogen-associated molecular pattern (PAMP) of LPS and signals through pattern recognition receptors (PRRs) of the host immune system. Lipid A is known to bind to the Toll-like receptor 4 (TLR4), which is a type of pattern recognition receptor (PRR), in many cell types, but especially in immune cells like microglia, monocytes, macrophages, dendritic cells and B cells.^{172–174} This interaction triggers a signaling cascade that activates the host immune response, leading to the production of pro-inflammatory cytokines (tumor necrosis factor α (TNF- α), interleukin (IL)-1 β , IL-6, etc.). This process is a crucial part of how the immune system detects and responds to acute Gram-negative bacterial infections.¹⁷⁵ However, in cases of prolonged LPS exposure (for instance due to

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dysbiosis of the gut microbiota), the immune system remains in an overactivated state.¹⁷⁶ As the immune cells (e.g. microglia) are continuously stimulated by LPS, they keep producing inflammatory cytokines, reactive oxidative species, and chemokines. Chronic exposure to LPS can further disrupt anti-inflammatory feedback loops, allowing inflammation to persist and creating a chronic low-grade pro-inflammatory microenvironment in affected tissues.¹⁷⁷

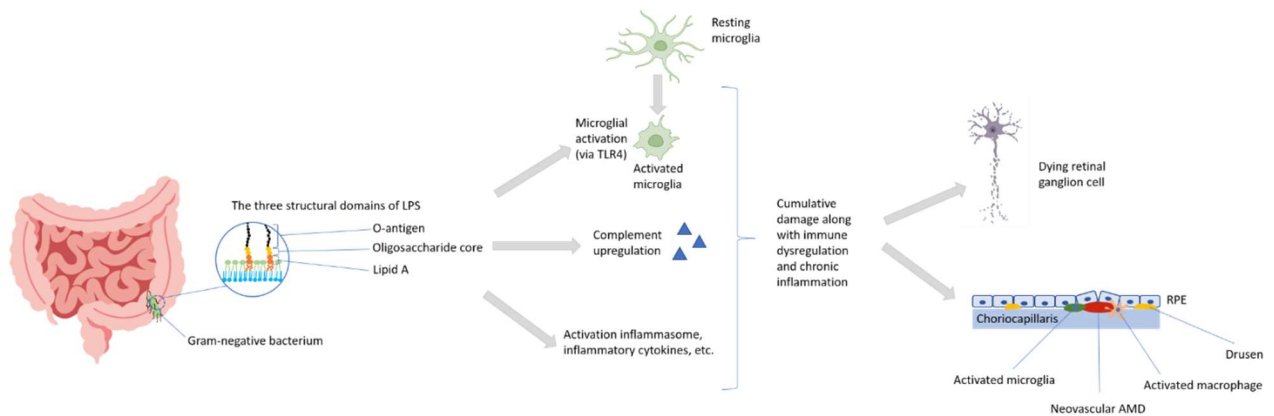


Figure 11. The gut - retina axis

Impaired gut microbiota leads to translocation of proinflammatory signaling including LPS into systemic circulation. Microglia are among the first cells to be activated via Toll-like receptors, which in turn induces the release of inflammatory cytokines. LPS: Lipopolysaccharides.

1.3.2 The impact of LPS on disease

1.3.2.1 The impact of LPS on systemic disease

LPS was first studied in humans primarily in the context of septic shock, a critical state of disease with mortality that can exceed 50 %.¹⁷⁵ In the context of septic shock and acute endotoxemia, Gram-negative bacteria are rapidly lysed and LPS is released into the bloodstream. LPS binds to TLR4 on immune cells, activating signaling pathways that stimulate the release of pro-inflammatory cytokines (such as TNF- α , IL-1, and IL-6). The massive release of cytokines is referred to as a "cytokine storm", which can damage tissues and organs.¹⁷⁸ The inflammatory mediators cause vasodilation and increased vascular permeability, leading to hypotension and potentially multi-organ failure.

Distinct from the acute response to LPS in gram negative infection and acute endotoxic septic shock, chronic exposure to LPS (chronic endotoxemia) has been implicated in the development of atherosclerosis and other cardiovascular diseases, where LPS-driven inflammation is thought to accelerate endothelial dysfunction and plaque formation in blood

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vessels.¹⁷⁹ Clinical data has further linked elevated plasma LPS levels to chronic low-grade inflammation seen in metabolic disorders such as obesity, type 2 diabetes, and metabolic syndrome.^{180–183} Higher levels of circulating LPS have also been implicated in the development and progression of non-alcoholic fatty liver disease, inflammatory bowel disease and chronic kidney disease.^{184–187}

1.3.2.2 The impact of LPS on eye disease

Mounting evidence indicate the involvement of LPS in the development of ocular conditions, including AMD and glaucoma.¹⁸⁸ In the eye, pro-inflammatory signaling via LPS occurs in microglia, perivascular macrophages, dendritic cells, photoreceptors and RPE cells and has been implicated in retinopathy of prematurity, diabetic retinopathy, AMD and glaucoma (**Figure 11**). Retinal explants stimulated by LPS showed a significant neuroinflammatory response characterized by upregulation of several cytokines and neuronal degeneration.¹⁸⁹ Low-dose LPS administration in glaucoma animal models resulted in enhancement of axonal degeneration and neuronal loss.¹⁹⁰ Also, increased neuronal apoptosis and evidence of microglial activation after LPS exposure has been reported.¹⁹¹ However, to our knowledge, no epidemiological study specifically analyzed exposure to LPS in age-related eye diseases.

1.3.3 Sources of chronic endotoxemia in human disease

1.3.3.1 Metabolic endotoxemia

The human gut microbiota consists of trillions symbiotic microorganisms living in a highly dynamic ecosystem.¹⁹² Besides facilitating digestion, the gut microbiota serves a variety of metabolic and supportive functions essential for physiological homeostasis, including maintenance of the intestinal barrier, participation in the intestinal immune system and generation of biologically active metabolites. As the gut is the primary reservoir for Gram-negative bacteria, it is the major source of LPS in humans. In recent years, a link between gut microbiota, diet and micronutrients in AMD has been described as the so-called “gut–retina axis”.^{193,194} Growing evidence also suggests the microbiome plays a critical role in glaucoma pathophysiology.¹⁸⁸

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Metabolic endotoxemia occurs when small amounts of LPS translocate from the gut into the bloodstream due to increased intestinal permeability ("leaky gut"). Many different factors are implicated to be involved in the leaky gut:

Ageing and diet affect the structure of the human gut microbiota, as well as its homeostasis with the host's immune system. In particular, high fat diets promote endotoxin-producing and sulphate-reducing bacteria, associated with mucus layer degradation, low-grade inflammation and insulin-resistance.^{196,197} Further, meals rich in saturated fats have been shown to transiently increase circulating LPS levels as fat can increase the absorption of LPS from the gut.^{198,199} Recent studies also showed that stress, alcohol, smoking, long-term use of nonsteroidal anti-inflammatory drugs, radiation and lack of physical activity have an impact on gut microbiota.^{200–205}

In particular, this increased permeability may result in elevated concentrations of antigens, pathogens and other proinflammatory substances, including lipopolysaccharide (LPS)-type endotoxins to pass through intestinal barrier into circulation.

1.3.3.2 Other sources of chronic endotoxemia

Aside from the gut, other significant sources of chronic endotoxemia include infections in the i) the oral cavity and ii) the respiratory tract.

- i) Oral cavity: Chronic infections in the oral cavity, such as periodontitis, are linked to an increased systemic load of LPS. Gram-negative bacteria like *Porphyromonas gingivalis*, found in periodontal disease, release LPS, which can enter the bloodstream through inflamed or damaged gum tissue. This can contribute to systemic inflammation and endotoxemia.
- ii) Respiratory tract: Gram-negative bacteria, such as *Pseudomonas aeruginosa*, are commonly involved in chronic respiratory infections. LPS from these bacteria can enter the bloodstream, especially in people with impaired lung function or compromised immune systems. Additionally, environmental and occupational exposures through inhalation of endotoxin-laden dust or aerosols can contribute to endotoxemia.

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Further, also other infections have been implicated in chronic endotoxemia including the urinary tract, skin, and chronic medical device-related infections. Each of these sources involves Gram-negative bacteria that produce LPS, which can enter the bloodstream and trigger systemic inflammation over time, even at low levels.

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1.4 Objectives

The overall objective of this thesis is to investigate the interplay between LPS exposure and the aging retina. In the framework of a population-based cohort with a follow-up of 15 years, I investigated the associations of LPS exposure with age related diseases and their morphologic changes in SD-OCT.

My working hypothesis concerned the proinflammatory role of LPS in the aging retina. In order to answer this question for AMD, I evaluated the relationship of plasma 3-hydroxy fatty acids (3-OH FAs), a proxy of total LPS burden, with incident early and advanced AMD (Article 1), taking into account potential confounders.

Taking advantage of cutting-edge ophthalmological imaging, I then studied the associations of 3-OH FAs with age-related morphologic changes of the retina. We assessed the potential associations of 3-OH FAs exposure with the longitudinal changes of the peripapillary retinal nerve fiber layer, a biomarker of retinal neurodegeneration, and a hallmark of glaucoma (Article 2).

After exporting and quality checking all macular SD-OCT scans performed within the frame of the Alienor study, I studied the longitudinal macular layer thickness changes of eight distinct and three compound layers in AMD, including their potential associations with 3-OH FAs (Article 3).

2 Methodology

2.1 Study design of the Alienor Study

2.1.1 Study cohort

All analysis performed within the frame of this thesis were based on the Alienor (Antioxydants, Lipides Essentiels, Nutrition et maladies OculaiRes) study, a subsample of the Bordeaux cohort of the Three-City (3C) study.^{118,206}

The 3C study is a multicenter population-based prospective cohort study initiated in 1999 to assess the vascular risk factors of dementia. The 3C study included community-dwelling individuals aged over 65 years randomly drawn from electoral rolls. Between 1999 and 2001, 9294 subjects were recruited in three French cities: Bordeaux (n = 2104), Dijon (n = 4931), and Montpellier (n = 2259). The Bordeaux cohort recruited subjects in 10 districts of Bordeaux and its suburbs, with a participation rate of 37 %. Each subject was monitored every two to three years, with a wide range of tests including cognitive assessment and cardiovascular investigations. The protocol of the 3C study was approved by the Consultative Committee for the Protection of Persons participating in Biomedical Research at Kremlin-Bicêtre University Hospital (Paris, France). All participants provided written informed consent.

During the 3rd follow-up of the 3C study (2006-2008), an ophthalmological examination was offered to all 1,450 subjects from Bordeaux still participating. A total of 963 (66.4 %) subject were thus enrolled into the Alienor cohort at baseline. Since their inclusion in 2006-2008, these individuals have been proposed an ophthalmological examination every two to three years in parallel with the 3C study (**Figure 12**). In 2024, the 6th and last ophthalmological follow-up visit of the cohort was completed, which correlates to up to 18 years of ophthalmological follow-up (<http://www.alienor-study.com/langue-english-1.html>, accessed on 24 July 2024).

The Alienor study was approved by the Ethics Committee of Bordeaux (Comité de Protection des Personnes Sud-Ouest et Outre-Mer III) in 2006 and followed the tenets of the Declaration of Helsinki. Informed consent of all participants of the Alienor Study was obtained after explanation of the nature and possible consequences of the study.

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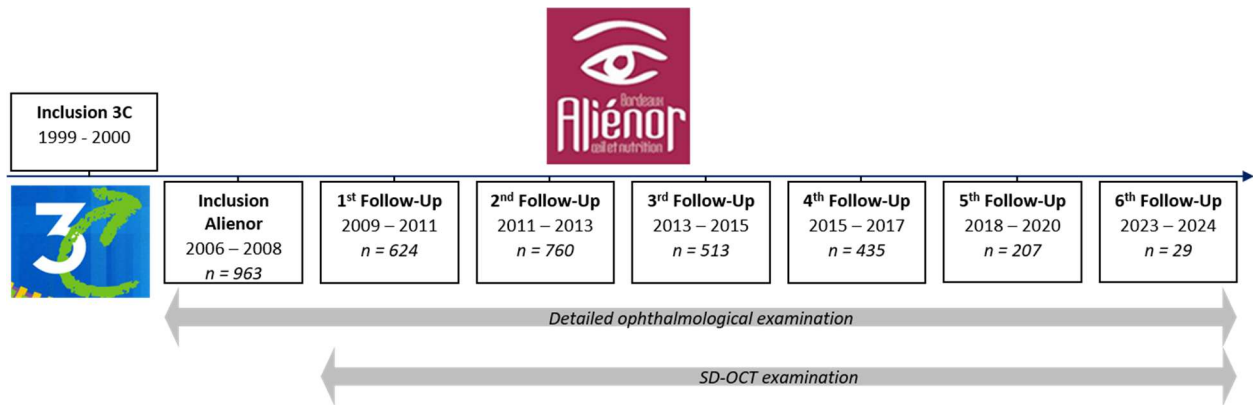


Figure 12. Design of the Alienor Study

The Alienor (*Antioxydants, Lipides Essentiels, Nutrition et maladies OculaiRes*) study consists of biennial eye examinations from 2006 until 2024. SD-OCT: spectral domain optical coherence tomography.

2.1.2 Study examinations

The Alienor study consisted of biennial eye examinations at the Department of Ophthalmology of the University Hospital of Bordeaux.

The study examination, lasting about an hour per participant, was carried out by specially trained orthoptists. Documented study visit data included:

- detailed ophthalmological history, using a standardized questionnaire,
- measurement of refractive error (Speedy K, Luneau, France),
- measurement of distance visual acuity with the usual correction and with optimal correction, using the ETDRS method,
- measurement of near visual acuity with the usual correction and with optimal correction (Parinaud test),
- two 45° retinal photographs (one centered on the macula, the other centered on the optic disc) using a high-resolution digital nonmydriatic retinograph (TRC NW6S; Topcon, Japan),
- measurement of intraocular pressure using an air tonometer (KT 800, Kowa, Japan),
- and from 2009, spectral domain optical coherence tomography (SD-OCT) examinations (Spectralis, Heidelberg Engineering, Heidelberg, Germany).

In addition, from the second to fifth follow-up, a home ophthalmological examination was offered to those unable to get to the University Hospital of Bordeaux. This examination, also

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carried out by an orthoptist, was similar to that performed at the hospital, with the exception of SD-OCT examination.

2.2 Ophthalmological Data

2.2.1 The AMD Grading scheme

As OCT examinations were not part of the initial Alienor study protocol, classification of AMD stages was primarily based on color fundus photography.

The AMD grading scheme followed the International Classification and Grading System by Bird et al., modified for drusen size, location and area according to the Multi-Ethnic Study of Atherosclerosis.^{118,207,208} Grading was performed from retinal photographs in duplicate by two trained graders and inconsistencies between the two graders were adjudicated by a retina specialist.^{42,118} Retinal photographs of each visit were classified in five exclusive stages:

- Stage 0: no AMD
- Stage 1: early AMD1, corresponding to large soft distinct drusen (> 125 microns in diameter) alone or pigment abnormalities (areas of hyperpigmentation and/or hypopigmentation (without visibility of choroidal vessels)) alone
- Stage 2: early AMD2, corresponding to large soft indistinct drusen (> 125 microns in diameter) and/or reticular drusen and/or large distinct drusen with pigment abnormalities,
- Stage 3: advanced atrophic AMD, defined as a discrete area of retinal depigmentation, 175 µm in diameter or larger, characterized by a sharp border and the presence of visible choroidal vessels
- Stage 4: advanced neovascular AMD, including serous or hemorrhagic detachment of the retinal pigment epithelium (RPE) or sensory retina, subretinal or sub-RPE hemorrhages, and fibrous scar tissue

In addition, from 2009 on, SD-OCT macular scans were interpreted for signs of advanced atrophic AMD and neovascular AMD (retinal atrophy, subretinal fluid, subretinal tissue, pigment epithelium detachment, intra-retinal fluid).

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Classification of advanced AMD (atrophic or neovascular) was performed by a retina specialist, based on all available information (ophthalmological history and treatments, retinal photographs, SD-OCT scans).

Incidence of early AMD was defined as the eye progressing from no AMD at baseline eye examination (2006–2008) to early AMD at any time-point during the study period (2006–2017). Incidence of advanced AMD was defined as the eye progressing from no or early AMD at baseline eye examination (2006–2008) to advanced AMD at any time-point during the study period. The date of occurrence of early/advanced AMD was calculated as the midpoint of the interval between the last visit without early/advanced AMD and the first visit with early/advanced AMD.

To increase the comparability of our data with clinical routine data, our group has started using the Beckman AMD classification,¹² with intermediate AMD defined as presence of large drusen and/or pigmentary abnormalities, in the absence of advanced AMD (atrophic and or neovascular).

2.2.2 Optical coherence tomography (OCT)

From the first follow-up visit (2009–2011), SD-OCT examinations of the macula and the optic nerve were added to the Alienor study protocol and are thus available from the 2nd eye visit. Thus, SD-OCT examinations are available at six time points from 2009 to 2024. The same OCT device was used at all follow-up visits. All SD-OCT assessments were performed by the same experienced technician. The standard mean keratometry value of 7.7 mm of the ophthalmic software and device was applied for SD-OCT measurements. The Spectralis OCT provides an automatic real-time (ART) function that adjusts for eye movement by combining multiple images of the same location to reduce noise and increase image quality.

For the peripapillary RNFL acquisition, the high-resolution protocol was employed. The RNFL thickness was calculated using the circle scan provided by the machine, which consisted of a 3.45-mm diameter circle scan centered on the optic disc. Correction for fovea-disc orientation is incorporated in the software. Global and sectorial (nasal and temporal quadrants, temporal-superior, nasal-superior, temporal-superior and nasal-inferior sectors) peripapillary RNFL thicknesses were automatically segmented and calculated (**Figure 13**).

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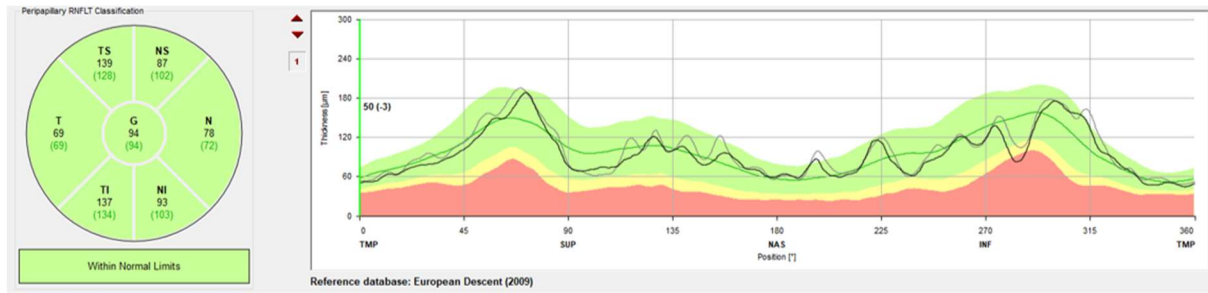


Figure 13. Peripapillary retinal nerve fiber layer thickness (RNFLT)
Example of RNFLT measurement by spectral-domain optical coherence tomography. Global peripapillary RNFLT was automatically segmented and calculated by the Heyex software.

For the macular cube acquisition, the following conditions were used: resolution mode: high speed; scan angle: 20°; number of B-scans: 19; pattern size: 20 × 15 degrees; and distance between B-scans: 236 µm. Single horizontal and vertical B-scan images (1536 A-scans) centered on the fovea were also performed.

The SD-OCT-layer segmentation was performed using the inbuilt automated segmentation algorithm of the Heidelberg Eye Explorer (HEYEX) on the macular volume scan. The accuracy and reproducibility of the inbuilt automated segmentation algorithm of the HEYEX has been previously reported.²⁰⁹

In the framework of this thesis, I exported all available SD-OCT data of the Alienor cohort between January and April 2024, using the HEYEX inbuilt “Thickness Map Data” batch-export function. This batch export function allows the export of more than one subject, but only one layer at a time. The output consists of an xls-file reporting the average thickness (in µm) of the respective layer in nine sectors (center, superior inner, temporal inner, inferior inner, nasal inner, superior outer, temporal outer, inferior outer and nasal outer) of the 6 mm diameter Early Treatment Diabetes Retinopathy Study (ETDRS) grid centered on the fovea.

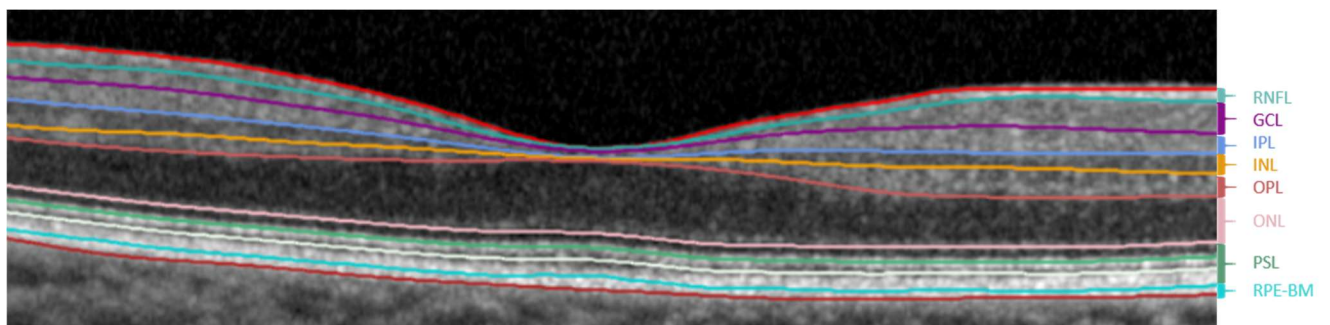
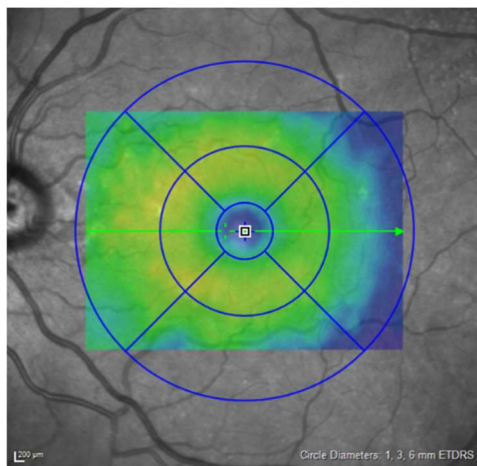


Figure 14. Segmented spectral domain optical coherence tomography (SD-OCT) scan
Example of an auto-segmented SD-OCT scan using the inbuilt automated segmentation of the Heidelberg Eye Explorer.

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Within the HEYEX software, using options, followed by preferences, the user can choose which layer to export. The HEYEX segmentation algorithm delineates the following macular layers: retinal nerve fiber layer (RNFL), ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL), and retinal pigment epithelium (RPE). Furthermore, the layers from RNFL to ONL are combined to inner retinal layers (IRL), the photoreceptors and the RPE to outer retina retinal layers (ORL), and all layers to total retinal layers (TRL) (**Figure 14**). An additional layer distinct from the above listed can be calculated by subtraction of the RPE from the ORL. This layer represents the photoreceptor inner and outer segments including the interdigitation zone and we will refer to it as photoreceptor segments layer (PSL) in the following.

As a recent cross-sectional study showed associations between retinal layer thicknesses and early AMD mainly in the central and inner ring (consisting of the superior inner, temporal inner, inferior inner and nasal inner sector) of the ETDRS grid (**Figure 15**),²¹⁰ we focused our analysis on those two circles. Moreover, the macular cube performed in the Alienor study



was too small to measure the layers in the outer ring. The mean thickness of the inner ring was determined by calculating the combined mean of the four inner sectors: superior inner, temporal inner, inferior inner, nasal inner.

Figure 15. The ETDRS grid

The ETDRS grid is overlaid on a confocal scanning laser ophthalmoscopy near-infrared reflectance image. The central ring, located at the center of the grid, has a diameter of 1 mm. Surrounding it is the inner ring, which is divided into four subfields and has a diameter of 3 mm. The outer ring is also divided into four subfields, and has a diameter of 6 mm.

Between January and April 2024, I exported all available SD-OCT data of the Alienor cohort and reviewed segmentation quality of all SD-OCT images of participants with missing SD-OCT data, retinal diseases and identified outliers (Linear Regression was applied to assess the agreement between measurements.) as well as signal strength lower than 15 dB.

Acquisitions with artefacts and segmentation errors were excluded from the analysis.

All SD-OCT derived variables were used as quantitative variables in the frame of this thesis.

Methodology

2.3 Lipopolysaccharide (LPS)- type endotoxins

Blood samples were obtained of 1,881 participants of the 3C study at baseline, including 902 (93.7 %) subjects of the Alienor study. All plasma measurements, including LPS-type endotoxins were assessed using blood samples collected during the 3C baseline visit (1999–2001) and subsequently stored in a bio-bank (serum, plasma and DNA) at –80 °C until determination.

The lacking assay standardization and the susceptibility of direct LPS exposure measurements to technical and measurement biases, has led to the development of several proxy methods to measure endotoxin levels.^{211–214} Recently, Pais de Barros and colleagues have established a method to quantify esterified 3-hydroxy (OH)-fatty acids (FAs) in plasma as a proxy of total plasma LPS burden using liquid chromatography tandem mass spectrometry.²¹⁵ This method measures the difference between the amounts of 3-OH-FAs in plasma after strong acidic hydrolysis and the amounts of free (i.e., non-LPS) 3-OH-FAs. The resulting circulating esterified 3-OH-FA level is a proxy of total plasma LPS burden. All esterified 3-OH-FA measurements were performed at the Plateforme de Lipidomique, INSERM UMR1231, Université de Bourgogne Franche Comté, Dijon, France and the detailed protocol can be found as Appendix 1.

3-OH FA measurements were standardized using the formula $Z = (x - \mu) / \sigma$, where Z is the standard score, μ the mean and σ the standard deviation of 3-OH FAs, respectively. The standardized 3-OH FA levels were recorded as quantitative variables.

Methodology

2.4 Polygenic risk score for AMD

Genotyping was performed by the French Centre National de Génotypage at 3C study baseline (1999–2001) using Illumina Human610-Quad BeadChips.²¹⁶ The present genetic risk score is based on the weighted risk score published by Fritsche and colleagues³. The score corresponds to the sum of the corresponding beta multiplied by the number of minor alleles for each single nucleotide polymorphism (SNP). The betas used are calculated from the fully conditioned odds ratios of Fritsche et al.^{3,42} Due to the high number of missing data for the three following SNPs: TRPM3 rs71507014, CNN2 rs67538026, and MMP9 rs142450006, these SNPs have been excluded from the risk score calculation. The present genetic risk score is thus based on 49 SNPs and was calculated for all participants who had available data for at least the five majors AMD-related genes (CFH rs10922109, CFH rs570618, C2 rs11603772, C3 rs2230199, and ARMS2 rs3750846).

The pathway-specific polygenic risk scores were calculated for the four main biological pathways: the complement system, lipid metabolism, and ECM remodeling using SNPs of the following genes²⁰: C2, C3, C9, CFH, CFI and TMEM97 for the complement pathway, ADAMTS9, COL4A3, COL8A1, SYN3 and VEGFA for the ECM pathway; ABCA1, APOE, CETP, LIPC for the lipid pathway; and ARMS2 for the ARMS2 pathway.

The polygenic risk scores were used as quantitative variable after Z score standardization.

Methodology

2.5 Other variables

2.5.1 Baseline characteristics

At the inclusion of the 3C study in 1999-2001, the subjects' socio-demographic characteristics were collected during a personal interview by means of a standardized questionnaire conducted by psychologists or trained nurses. These general data included date of birth, sex, residential address, physical activity, as well as the type and frequency consumption of tobacco and alcoholic beverages.

Sex was coded as categorical variable with two classes: male and female.

The baseline residential address was used to calculate the social deprivation index, a socio-economic index based on the French EDI (European deprivation index) adapted to elderly,²¹⁷ which was used as a quantitative variable.

Physical activity was used in our analyses as a categorical variable with three classes: low intensity (hardly no physical activity), moderate intensity (practicing physical activity with little perspiration) and high intensity (practicing physical activity with perspiration).

Smoking was used in our analyses as a categorical variable with three classes: non-smoker, < 20 pack-years and ≥ 20 pack-years (pack-years = number of cigarettes per day)/20 * number of years of smoking).

2.5.2 Blood samples and medical variables

Fasting blood glucose, cholesterol (total cholesterol, low-density lipoprotein (LDL)-cholesterol and HDL-cholesterol) and triglycerides were measured at the Biochemistry Laboratory of the University Hospital of Dijon, France using routine techniques and recorded as quantitative variables.

Diabetes was assessed as fasting blood glucose ≥ 7 mmol/L or diabetes medication and recorded as binary variable.

History of cardiovascular disease and vascular risk factors (hypertension: blood pressure > 140/90 mm Hg or antihypertensive medication, hypercholesterolemia, diabetes, stroke, angina pectoris, myocardial infarction, arteritis, cardiac and vascular surgery) were recorded as binary variables.

Methodology

Systolic and diastolic blood pressure, weight and height were measured and recorded as quantitative variables.

The body-mass-index (BMI) was calculated by the formula: $BMI = \text{weight [kg]} / \text{height[m]}^2$ used in our analyses as a categorical variable with four classes: underweight [0,18.5), normal [18.5,25), overweight [25,30) and obese [30,59].

Medication intake was recorded and grouped according to the Anatomical Therapeutic Chemical (ATC) classification system. The variables number of drugs used, anti-inflammatory medication and lipid-lowering medication were based on the ATC-grouped medication intake. Number of drugs used was recorded as ordinal variable and both anti-inflammatory medication and lipid-lowering medication as binary variables.

2.5.3 Mediterranean diet score

The Mediterranean diet score was calculated using a 148-items validated food frequency questionnaire (FFQ) administered at home by dietitians and the MEDI-LITE score developed by Sofi et al.²¹⁸ As previously described, this score includes the frequency of consumption of nine components: high consumption of fruits, vegetables, legumes, cereals, fish and olive oil, moderate consumption of dairy products and alcohol, and low consumption of meat were defined as beneficial in this score.²¹⁹ Consumption frequency thresholds were estimated by Sofi and colleagues from literature.²¹⁸ The final score indicates the degree of adherence to the Mediterranean diet, ranging from 0 (poor adherence) to 18 (highest adherence). The Mediterranean diet score was used as quantitative variable.

Methodology

2.6 Statistical analysis

R software version 4.1.1 – 4.4.1 (R Foundation for Statistical Computing, Vienna, Austria) was used for all statistical analyses.

Throughout the work on this thesis, all data were first analyzed descriptively and then mainly two statistical models were employed. Thus, in the following, I will first address the descriptive data analysis and the logistic regression models, and then focus on the two main models used in this thesis, the proportional hazard Cox models and the multivariable mixed linear regression models.

2.6.1 Descriptive data analysis

For each objective of this study, the studied cohort was first analyzed descriptively using appropriate absolute and relative frequencies for categorical data and medians and quartiles / means and standard deviations for continuous data. Exploratory analyses were performed by means of the Student's t-test (continuous data), the Wilcoxon test (non-Gaussian variables/ multi-categorical variables) and the McNemar's χ^2 test (categorical data).

2.6.2 Logistic regression models

The associations of both AMD and glaucoma at the baseline eye examination (2006-2008) and circulating level of esterified 3-OH FAs were evaluated using logistic regression models.

First, I used a classic logistic model, only including one eye (the worse eye) of each subject. Separate analyses were conducted for early AMD and advanced AMD, and for several characteristic features of early AMD (in particular foveal drusen and pigmentary abnormalities).

Second, in order to take into account the data from both eyes and their correlation, I used a marginal model, the Generalized Estimating Equation (GEE²²⁰) logistic model, often used in ophthalmological studies. Separate analyses were conducted for early AMD and advanced AMD, and for several characteristic features of early AMD (in particular foveal drusen and pigmentary abnormalities).

Methodology

2.6.3 Proportional hazard Cox modelling

Proportional hazard Cox modelling was used to model the associations of esterified 3-OH FAs with incident (and progression) of AMD using time since baseline or age as time scale and delayed entry. Using age as time scale adjusts automatically for the confounding effect of age and thus allows for a more appropriate adjustment of age of the elderly than the classical Cox models based on the time from entry in the study.²²¹

The individual eye was used as the unit of analysis. To take into account intra-individual correlation between eyes of one participant, the “cluster” term was included in the using Cox proportional hazards models in R (R Foundation for Statistical Computing, Vienna, Austria):

Using time from baseline as time scale:

```
coxph(Surv(time, status) ~ sex + age + LPS_s + cluster (subject), data)
```

Using age as time scale:

```
coxph(Surv(age_inclusion, age_amd, status) ~ sex + age + LPS_s +  
cluster (subject), data)
```

All conditions for the application of the Cox model were verified. To account for potential nonlinear effects of quantitative variables, we used penalized splines with four degrees of freedom (pspline function in the Coxph function of R). The proportional hazard assumption was checked using Schoenfeld residuals.

2.6.4 Multivariable mixed linear regression models

Multivariable mixed linear regression models were used to assess longitudinal associations of esterified 3-OH FAs with change in retinal morphology over time. The individual eye was used as the unit of analysis, to take into account intra-individual correlation between eyes of one participant the data of the individual eye was nested within each subject:

```
(1|subject/eye)
```

Methodology

Interaction between 3-OH FAs and time was used to account for longitudinal effects:

```
lmer(thickness ~ sex + age_inclusion + LPS_s * time +  
(1|subject/eye), data)
```

All conditions for the application of the linear mixed model were verified. The linearity of quantitative variables was investigated using restricted cubic spline functions.

2.6.5 Multivariate adjustment and Imputation

Each model was first adjusted for sex and age and then further adjusted for relevant potential confounders, identified using a directed acyclic graph after literature research. As some data on these identified variables were missing, we performed multivariate imputation by chained equations (MICE), assuming that missing data were Missing At Random (MAR).²²² The missing values were imputed based on the observed values for a given individual and the relations observed in the data for other participants. A total of 10 cycles was performed to obtain the final imputed dataset for missing potential confounders.

2.6.6 Correction for multiple testing

To address multiple testing, the Benjamini-Hochberg procedure was applied for false discovery rate (FDR) correction.²²³ The Benjamini-Hochberg procedure controls the FDR, ensuring that, on average, the proportion of false discoveries among significant results remains below the specified threshold. It is less conservative than methods like the Bonferroni correction, allowing for more discoveries while still controlling the error rate.

The Benjamini-Hochberg procedure adjusts p-values in a stepwise manner aiming to limit the proportion of false positives: After p-values are assigned ranks according to their ascending value, adjusted p-values were calculated using the following formula, where:

$$p_{adjusted(i)} = \min \left(1, \min_{k \geq i} \left(\frac{m}{k} \cdot p_{(k)} \right) \right)$$

- adjusted $p_{(i)}$ represents the adjusted p-value for the i-th ranked p-value,
- $p_{(k)}$ represents the k-th ranked p-value and
- m represents the total number of hypotheses being tested.

Methodology

Statistical significance was determined using an FDR-corrected P value (P FDR) of less than 0.05.

2.6.7 Data handling

Data acquired or analyzed in this project was pseudonymized and handled according to GCP regulations. Data handling was accurately and safely performed with respect to reviewing and retaining electronic source data.

3 Results

3.1 Association of LPS with AMD

3.1.1 Introduction

AMD is a multi-factorial and complex disease and the leading cause of vision loss among older adults in developed countries.¹ Global pooled data of people between 45 and 85 years estimate the worldwide prevalence of AMD at 8.7 %.²⁴

Multiple factors play important roles in the development of AMD, whereas the underlying pathological mechanisms are not yet fully understood. Although risk factors such as smoking, diet, cardiovascular diseases, and genetics are known, the exact mechanisms of AMD are not fully understood. Key pathogenetic factors include oxidative stress, immune dysregulation, and low-grade chronic inflammation.¹

Recent research has linked the human microbiota to retinal health. The human microbiota, consisting of trillions of microorganisms, affects digestion, immune function, and barrier maintenance.^{224,225} Diet and age influence gut microbiota composition. High-fat diets promote inflammation and insulin resistance by increasing endotoxin-producing bacteria. Stress, alcohol, smoking, radiation, and physical inactivity also impact gut microbiota.¹⁹⁶ Microbial dysbiosis result in increased intestinal permeability, allowing pro-inflammatory substances like lipopolysaccharides (LPS) into the bloodstream. LPS is a natural component of Gram-negative bacteria and has been implicated to induce and amplify chronic inflammation in various diseases, including AMD.^{224,226} Inflammatory damage may lead to the breakdown of the blood-retina barrier, resulting in a breach of the retinal immune privilege. As the breakdown of the blood-retina barrier is one of the hallmarks of AMD, it can be postulated that changes will also reflect systemically.

Our analysis was performed within the prospective population-based Alienor study, which is characterized by a detailed standardized ophthalmological examination and long follow-up time.

The aim of Article 1 was to explore the associations between LPS and the incidence of AMD in the subjects of the Alienor study.

Results

3.1.2 Article 1

Association of Age-Related Macular Degeneration with a Blood Biomarker of Lipopolysaccharide, a Gut Bacterial Proinflammatory Toxin

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PURPOSE. Chronic inflammation, immune dysregulation, and oxidative stress are major drivers of age-related macular degeneration (AMD) pathogenesis. Lipopolysaccharide (LPS) is a potent proinflammatory toxin originating from gut bacteria. We assessed the association of a blood biomarker of LPS exposure with incident AMD.

METHODS. The Alienor Study is a prospective population-based study, including 963 residents of Bordeaux (France), aged 73 years or more at baseline. Esterified 3-hydroxy fatty acids (3-OH FAs) were measured from blood samples as a proxy of LPS burden. AMD was graded from color retinal photographs and spectral domain optical coherence tomography, performed every two years from 2006 to 2017. Cox proportional hazards models were used to estimate associations of between esterified 3-OH FAs, using 722 eyes at risk for incident early AMD and 981 eyes at risk for incident advanced AMD.

RESULTS. Higher esterified 3-OH FAs were associated with incident early AMD after adjusting for age and gender (hazard ratio [HR] = 1.21 for 1 standard deviation [SD] increase; 95% confidence interval [CI], 1.01–1.45; $P = 0.04$) but not with incident advanced AMD (HR = 1.03 for 1 SD increase; 95% CI, 0.73–1.45; $P = 0.86$). These associations remained stable after multivariate adjustment and imputation for missing covariates (early AMD HR = 1.22 for 1 SD increase; 95% CI, 1.01–1.46; $P = 0.04$; advanced AMD HR = 0.98 for 1 SD increase; 95% CI, 0.69–1.38; $P = 0.91$).

CONCLUSIONS. This study evidenced an association between higher esterified 3-OH FAs and incident early AMD, suggesting that exposure to LPS may be involved in the early pathophysiological processes of AMD.

Keywords: age-related macular degeneration, systemic inflammation, LPS, clinical (human) or epidemiologic studies: risk factor assessment

Age-related macular degeneration (AMD) is the leading cause of blindness among the elderly population in the developed world. Although large epidemiological studies have identified risk factors such as cigarette smoking, nutritional factors, cardiovascular diseases, and genetic markers, the underlying pathological mechanisms are not fully understood.^{1,2} Contributing factors in AMD pathogenesis are oxidative stress, along with immune dysregulation and chronic inflammation, particularly persisting low-grade inflammation.^{3,4}

In recent years, a link between gut microbiota and the retina has been described as the so-called “gut-retina axis.”^{5,6} The human gut microbiota consists of trillions symbiotic micro-organisms living in a highly dynamic ecosystem.⁷ Besides facilitating digestion, the gut microbiota serve a variety of metabolic and supportive functions essential for physiological homeostasis, including maintenance of the intestinal barrier, participation in the intestinal immune system, and generation of biologically active metabolites.

Diet and age are known to influence the composition of the gut microbiota. In particular, high-fat diets promote endotoxin-producing and sulphate-reducing bacteria, associated with mucus layer degradation, low-grade inflammation, and insulin-resistance.^{8,9} Recent studies also showed that stress, alcohol, radiation, and lack of physical activities have an impact on gut microbiota.^{10–12} Alterations in the gut microbiota impair the integrity of intestinal barrier, leading to increased intestinal permeability. This increased permeability allows antigens, lipopolysaccharides (LPS), pathogens, and other proinflammatory substances to pass through intestinal barrier into circulation.

As a component of the outer membrane of Gram-negative bacteria, LPS is naturally found in the human gut microbiota. LPS is a large glycolipid consisting of three structural domains: a core oligosaccharide, an O-antigen polysaccharide and Lipid A (Fig. 1).^{13,14} Two molecules of 3-hydroxy (3-OH) fatty acids (FAs) are esterified to the glucosamine backbone of Lipid A, and two are attached in amide

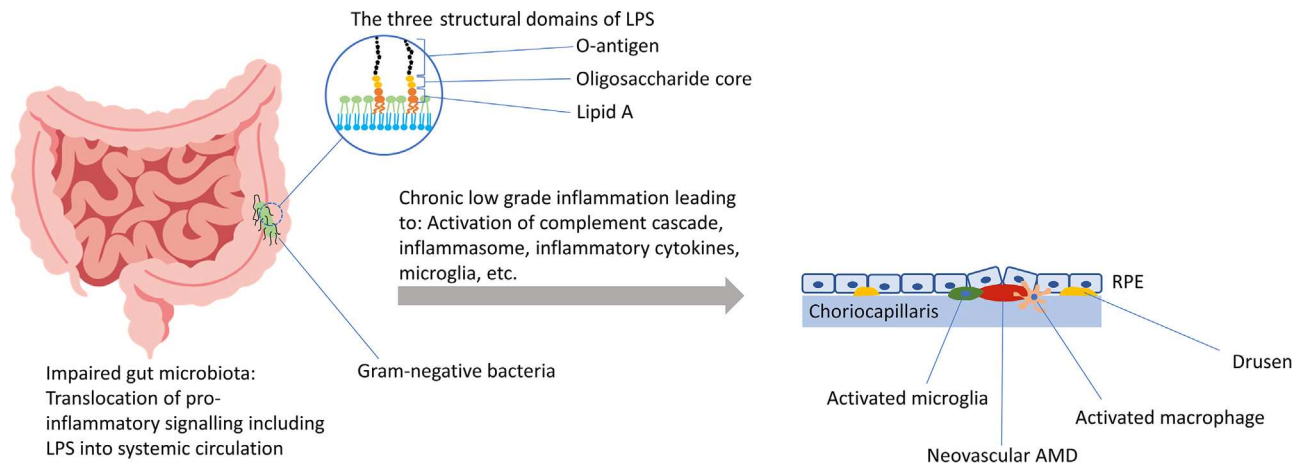


FIGURE 1. The “gut-retina axis” in AMD: Potential mechanisms that link impaired gut microbiota and LPS with the development and progression of AMD.

linkage.¹⁵ Lipid A acts as the pathogen-associated molecular pattern of LPS. When LPS concentrations increase, it acts as a potent immune activator and TLR4 ligand.^{16–18} Its inflammatory potential has been implicated in the development of multiple disease states, including insulin resistance, obesity, augmented cardiovascular risk, and fatty liver disease.^{19–22}

In the eye, proinflammatory signaling via LPS occurs in microglia, perivascular macrophages, dendritic cells, photoreceptors, and retinal pigment epithelium (RPE) cells and has been implicated in retinopathy of prematurity, diabetic retinopathy, and AMD (Fig. 1).^{5,23–27} Retinal explants stimulated by LPS showed a significant neuroinflammatory response characterized by upregulation of several cytokines and neuronal degeneration.²⁷ Also, increased neuronal apoptosis and evidence of microglial activation after LPS exposure have been reported.²⁸

Thus there is a substantial body of evidence pointing to the involvement of LPS-induced low-grade inflammation in the development of AMD. The mentioned *in vitro* studies are great models of disease, identifying pathways and links between exposure and disease outcome. Still, data from prospective human studies are needed to analyze long-term effects of chronic exposure.

Thus, in this study we assessed the association of a blood biomarker of LPS exposure with incident early and advanced AMD in the framework of a population-based cohort study of French older adults.

METHODS

The Alienor Study (Antioxydants, Lipides Essentiels, Nutrition et maladies Oculaires) is an ongoing prospective population-based study initiated in 2006 to assess the associations of age-related eye diseases with nutritional factors (in particular antioxidants, macular pigment, and fatty acids) and other risk factors (genetic susceptibility, environmental, and vascular factors, etc.).²

Study Cohort

Subjects of the Alienor Study were initially recruited from the Three City (3C) Study cohort of Bordeaux (France), which enrolled randomly selected individuals from electoral rolls

from 1999 to 2001.²⁹ Both the 3C Study cohort and the Alienor Study cohort have been described in detail elsewhere.^{2,29,30}

The Alienor Study included 963 residents of Bordeaux, aged 73 years or more at baseline (2006–2008). Participants underwent biennial examinations at the Department of Ophthalmology of the University Hospital of Bordeaux during the study period from 2006 to 2017 (four follow-up visits, <http://www.alienor-study.com/langue-english-1.html>, accessed on September 8, 2023). Of the 963 Alienor participants, 624 were reexamined at the first follow-up (2009–2010), 614 at the second follow-up visit (2011–2012), 513 at the third follow-up visit (2013–2015), and 435 at the fourth follow-up visit (2015–2017).

The Alienor Study followed the tenets of the Declaration of Helsinki and was approved by the Ethical Committee of Bordeaux (Comité de Protection des Personnes Sud-Ouest et Outre-Mer III, code 2006/10) in May 2006. Informed consent of all participants of the Alienor Study was obtained after explanation of the nature and possible consequences of the study.

Imaging and AMD Grading

Each Alienor Study visit included 45° nonmydriatic color retinal photographs using a high-resolution digital nonmydriatic retinograph (TRC NW6S; Topcon, Japan). From the first follow-up visit (2009–2011), also spectral-domain optical coherence tomography (SD-OCT) examinations of the macula and the optic nerve were performed (Spectralis; Heidelberg Engineering, Heidelberg, Germany). All SD-OCT assessments were performed by the same experienced technician. For the macular thickness acquisition, the following conditions were used: resolution mode: high speed; scan angle: 20°; number of B-scans: 19; pattern size: 20 × 15 degrees; and distance between B-scans: 236 μm. A single horizontal and vertical B-scan image (1536 A-scans) centered on the fovea was also performed.

As previously described, the AMD grading scheme followed the International Classification and Grading System by Bird et al., modified for drusen size, location and area according to the Multi-Ethnic Study of Atherosclerosis.^{2,31,32} Grading was performed from retinal photographs in duplicate by two trained graders and inconsistencies between

the two graders were adjudicated by a retina specialist.^{2,30} Early signs of AMD included large drusen (>125 microns in diameter), reticular pseudodrusen and pigmentary abnormalities (areas of hyperpigmentation and/or hypopigmentation (without visibility of choroidal vessels)). Neovascular AMD included serous or hemorrhagic detachment of the RPE or sensory retina, subretinal or sub-RPE hemorrhages, and fibrous scar tissue. Geographic atrophy was defined as a discrete area of retinal depigmentation, 175 μ m in diameter or larger, characterized by a sharp border and the presence of visible choroidal vessels. In addition, SD-OCT macular scans were interpreted for signs of advanced AMD (retinal atrophy, subretinal fluid, subretinal tissue, pigment epithelium detachment, intra-retinal fluid).

Finally, presence of large drusen and/or pigmentary abnormalities and/or reticular pseudodrusen were defined as early AMD (in the absence of advanced AMD), which corresponds to the definition of intermediate AMD in more recent classifications.³³ Classification of advanced AMD (atrophic or neovascular) was performed by a retina specialist, based on all available information (ophthalmological history and treatments, retinal photographs, SD-OCT scans).

Incidence of early AMD was defined as the eye progressing from no AMD at baseline eye examination (2006–2008) to early AMD at any time-point during the study period (2006–2017). Incidence of advanced AMD was defined as the eye progressing from no or early AMD at baseline eye examination (2006–2008) to advanced AMD at any time-point during the study period. The date of occurrence of early/advanced AMD was calculated as the midpoint of the interval between the last visit without early/advanced AMD and the first visit with early/advanced AMD.

Assessment of LPS-Type Endotoxins

All plasma measurements, including LPS-type endotoxins were determined from blood samples collected at the 3C baseline visit (1999–2001) and stored at -80°C until determination.

As direct measurement of LPS exposure is prone to technical and measurement biases,^{34–37} esterified 3-hydroxy fatty acids (3-OH FAs) in plasma were measured as a proxy of total plasma LPS burden using liquid chromatography tandem mass spectrometry (LC-tandem MS). This method has been established and previously described by Pais de Barros and colleagues.^{38,39} Briefly, the difference between the amounts of 3-OH FAs in plasma after strong acidic hydrolysis and the amounts of free (i.e., non-LPS) 3-OH FAs without strong acidic hydrolysis is calculated. The resulting circulating plasma esterified 3-OH FA levels are a proxy of total plasma LPS burden.

Other Variables

Polygenic risk score: Genotyping was performed by the French Centre National de Génotypage at 3C study baseline (1999–2001) using Illumina Human610-Quad BeadChips.⁴⁰ The present genetic risk score is based on the weighted risk score published by Fritsche and colleagues and was described in detailed elsewhere.^{30,41}

Mediterranean diet score: The Mediterranean diet score was calculated using a 148-items validated food frequency questionnaire (FFQ) administered at home by dietitians and the MEDI-LITE score developed by Sofi et al.⁴² As previously

described, this score includes the frequency of consumption of nine components: High consumption of fruits, vegetables, legumes, cereals, fish and olive oil, moderate consumption of dairy products and alcohol, and low consumption of meat were defined as beneficial in this score.⁴³ Consumption frequency thresholds were estimated by Sofi and colleagues from literature.⁴² The final score indicates the degree of adherence to the Mediterranean diet, ranging from 0 (poor adherence) to 18 (highest adherence).

Statistical Analyses

Associations of plasma esterified 3-OH FAs with AMD were estimated using Cox proportional hazards models with age as time scale and delayed entry. Using age as time scale adjusts automatically for the confounding effect of age and thus allows for a more appropriate adjustment of age of the elderly than the classical Cox models based on the time from entry in the study.⁴⁴

The individual eye was used as the unit of analysis. To take into account intra-individual correlation between eyes of one participant, the “cluster” term was included in the using Cox proportional hazards models in R (R Foundation for Statistical Computing, Vienna, Austria).

Imputation was performed using multivariate imputation by chained equations (MICE), which assumes that missing data were Missing At Random (MAR). The missing values were imputed based on the observed values for a given individual and the relations observed in the data for other participants.⁴⁵ For the fraction of missing/imputed data see Supplementary Table S1. A total of 10 cycles was performed to obtain the final imputed dataset for missing potential confounders. Multivariate adjustment was performed after imputation. Model 1 was adjusted for gender, model 2 was further adjusted for smoking status, plasma high-density lipoprotein (HDL) cholesterol, plasma low-density lipoprotein (LDL) cholesterol, plasma triglycerides, diet quality and AMD polygenic risk score and model 3 was further adjusted for number of drugs used, anti-inflammatory medication and lipid-lowering medication. Variables retained in model 2 were factors associated with the incidence of AMD and/or with LPS, in our study or in the literature.

R software version 4.2.3 (R Foundation for Statistical Computing, Vienna, Austria) was used for statistical analyses.

RESULTS

Characteristics of the Studied Sample

Of 1926 eyes of 963 participants, which were included in the Alienor Study, 70 eyes of 43 participants had already developed advanced AMD at baseline. In addition, 616 eyes of 385 participants had no gradable baseline and/or no gradable follow-up examination, leaving 1240 eyes of 696 participants at risk to develop incident advanced AMD (Fig. 2). Furthermore, 231 eyes of 133 participants were excluded because of missing data for plasma esterified 3-OH FA levels. Using Tukey's interquartile range criterion, we identified an additional 28 eyes of 15 participants as outliers (Supplementary Fig. S1). Therefore 981 eyes of 548 participants were included for further analyses of incident advanced AMD. For the sub-cohort of incident early AMD, the same study sample as for incident advanced AMD was used, after exclusion of participants with early AMD at baseline. Thus, of 981 eyes of

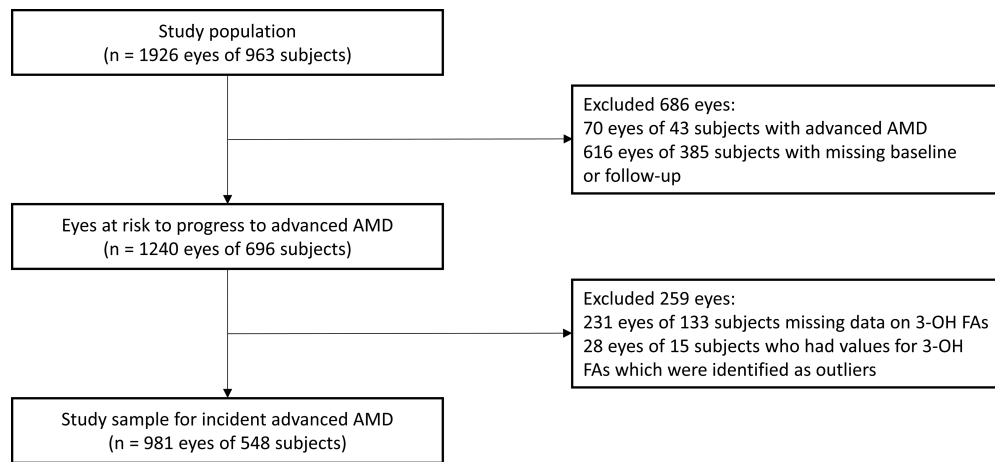


FIGURE 2. Flow chart showing selection of participants for analyses.

TABLE 1. Baseline Sociodemographic, Lifestyle, Medical, and Genetic Characteristics According to Participants Included and Non-Included in the Statistical Analyses: Alienor Study, 2006–2017

Characteristics	Included (n = 548)	Non-Included (n = 415)	P Value
Age (yr), mean $\pm$ SD	79.86 $\pm$ 4.46	80.57 $\pm$ 4.38	0.01
Gender			0.10
Men	196 (35.77%)	171 (41.20%)	
Women	352 (64.23%)	244 (58.80%)	
Smoking (pack-year)	n = 546	n = 405	0.12
Never smoker	363 (66.48%)	251 (61.98%)	
<20 years	100 (18.32%)	78 (19.26%)	
$\geq$ 20 years	83 (15.20%)	76 (18.76%)	
Mediterranean diet score, mean $\pm$ SD	10.59 $\pm$ 2.03	10.39 $\pm$ 2.23	0.18
Number of drugs used	4.00 $\pm$ 2.53	4.13 $\pm$ 2.64	0.45
Anti-inflammatory medication	84 (15.33)	75 (18.07)	0.29
Lipid-lowering medication	190 (34.67)	119 (28.67)	0.06
Plasma lipids (mmol/L)			
Total cholesterol	5.83 $\pm$ 0.94	5.71 $\pm$ 1.03	0.09
LDL cholesterol	3.67 $\pm$ 0.82	3.55 $\pm$ 0.89	0.03
HDL cholesterol	1.60 $\pm$ 0.39	1.58 $\pm$ 0.41	0.55
Triglycerides	1.20 $\pm$ 0.54	1.28 $\pm$ 0.69	0.07
Genetic risk score	0.28 $\pm$ 1.20	0.37 $\pm$ 1.20	0.31

HDL, high-density lipoprotein; LDL, low-density lipoprotein; SD, standard deviation.

548 participants, 259 eyes of 194 participants who already had early AMD at baseline were excluded, leaving 722 eyes of 446 participants for the analyses of incident early AMD.

Among the 548 included participants, mean age at baseline examination was 79.9 years ($\pm$ 4.5), of which 352 (64.2%) were female (Table 1). Included participants tended to be slightly younger and have higher LDL cholesterol (3.67 ± 0.82 vs. 3.55 ± 0.89) than the nonincluded participants of the Alienor Study. No significant difference was observed for other sociodemographic, lifestyle, medical, or genetic data. During a median follow-up time (censoring time)⁴⁶ of 7.3 years (range, 0.7 to 10.4), 195 (27.0%) eyes developed incident early AMD and 72 (7.3%) eyes incident advanced AMD.

Multivariate Associations Between Esterified 3-OH FAs in Plasma and the Incidence of AMD

Associations of esterified 3-OH FAs in plasma with incident early and advanced AMD are shown in Table 2. After adjustment for age (as the timescale) and gender, higher

plasma esterified 3-OH FAs was significantly associated with a higher risk of incident early AMD (hazard ratio [HR] for 1 standard deviation [SD] increase = 1.21; 95% confidence interval [CI], 1.01–1.45; $P = 0.04$). No significant association was observed between plasma esterified 3-OH FAs and incident advanced AMD (HR = 1.03 for 1 SD increase; 95% CI, 0.73–1.45; $P = 0.86$).

The association between plasma esterified 3-OH FAs and incident early AMD remained stable after multivariate adjustment and imputation for missing covariates (HR = 1.22 for 1 SD increase; 95% CI, 1.01–1.46; $P = 0.036$). As shown in Figure 3, the hazard of incident early AMD increased with esterified 3-OH FAs in plasma, after multivariate adjustment. The hazard function is represented using p-spline with 4 degrees of freedom, showing a continuous increase of the association between plasma esterified 3-OH FAs and incident early AMD to about the mid of the fourth quartile, followed by a steeper increase afterward.

In multivariable analyses, esterified 3-OH FAs in plasma were not associated with incident advanced AMD (HR = 0.98 for 1 SD increase; 95% CI, 0.69–1.38; $P = 0.91$).

TABLE 2. Associations between Esterified 3-OH FAs and Incidence of AMD: Alienor Study, 2006–2017

	Early AMD		Advanced AMD	
	No. of Eyes	Mean $\pm$ SD for 3-OH FAs	No. of Eyes	Mean $\pm$ SD for 3-OH FAs
Non-Incident AMD	527	254.6 $\pm$ 72.0	909	256.5 $\pm$ 74.0
Incident AMD	195	266.2 $\pm$ 77.5	72	256.9 $\pm$ 80.4
	HR (95% CI)	P Values	HR (95% CI)	P Values
Model 1 [*]	1.21 (1.01–1.45)	0.04	1.03 (0.73–1.45)	0.86
Model 2 [†]	1.22 (1.01–1.46)	0.04	0.98 (0.69–1.38)	0.91
Model 3 [‡]	1.21 (1.01–1.46)	0.04	1.00 (0.71–1.40)	0.98

HDL, high-density lipoprotein; LDL, low-density lipoprotein; SD, standard deviation; HR, hazard ratio.

^{*} Model 1, HR was estimated using Cox proportional model adjusted for gender. HR for 1 SD increase.

[†] Model 2, HR was estimated using Cox proportional model adjusted for gender, smoking, HDL cholesterol, LDL cholesterol, triglycerides, diet quality and AMD polygenic risk score. HR for 1 SD increase.

[‡] Model 3, HR was estimated using Cox proportional model adjusted for gender, smoking, HDL cholesterol, LDL cholesterol, triglycerides, diet quality, AMD polygenic risk score, number of drugs used, anti-inflammatory medication and lipid-lowering medication. HR for 1 SD increase.

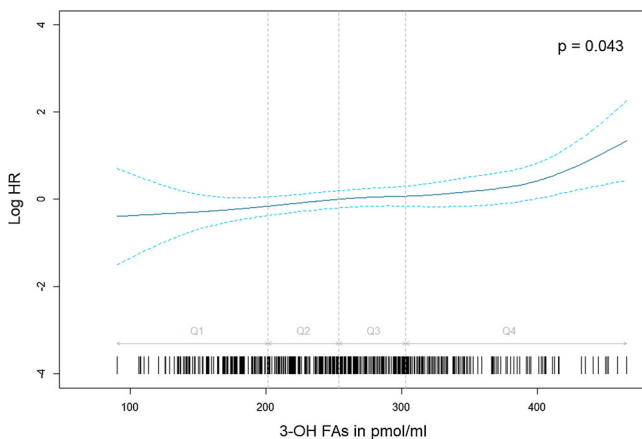


FIGURE 3. Association between 3-OH FAs and incidence of early AMD by eye adjusted for age, gender, smoking status, HDL cholesterol, LDL cholesterol, triglycerides, genetic risk score, and Mediterranean diet score. Data from the Alienor Study 2006–2017 ($n = 722$). All quantitative variables were modelled using p-spline with four degrees of freedom in the Cox model.

Finally, we performed sensitivity analyses by excluding participants with less than one year of follow-up (12 participants for the analysis of advanced AMD and seven participants for the analysis of early AMD). The results were similar to the main analyses (data not shown).

DISCUSSION

In this cohort of French older adults, higher baseline levels of 3-OH FAs, a proxy of plasma LPS exposure, were significantly associated with the incidence of early AMD. Because this gut endotoxin has proinflammatory properties, these results add to the evidence regarding the role of inflammation in AMD and further document emerging evidence of the gut-retina axis.

To our knowledge, this prospective population-based study is the first to investigate the relationship between plasma endotoxin exposure and the incidence of AMD.

However, many *in vitro* and animal studies underline the influence of plasma endotoxin exposure on AMD: Using primary human RPE cultures and ARPE19 cells, Leung et al.⁴⁷ demonstrated that LPS induced RPE-cytotoxicity via proinflammatory autocrine signaling of interleukin-6 and

interleukin-8, implying the role of LPS in the progression of macular pathologies.

In neovascular *in vivo* AMD models, both significant reduction of choroidal neovascularization (CNV) size with LPS treatment and exacerbation of CNV have been described. Specifically, Feng et al.⁴⁸ described promotion of CNV lesions after intravitreal LPS pretreatment (10 μ g; 10 ng/mL) one day before laser irradiation via TLR4 activation and subsequent upregulation of the expression of CXC chemokine receptors 4 and 7 in choroid-retinal endothelial cells. In line with this observation, also an enhancement of proinflammatory microglia/macrophages status by intravenously injected LPS (1 mg/kg) was observed to correlate with larger CNV leakage areas in the early onset of laser-induced CNV.²⁵ By contrast, it has been shown that low-dose (20 μ g) intraperitoneal LPS treatment before laser-induced CNV treatment suppresses CNV via interleukin-10 secretion by peritoneal macrophages in mice.²⁴ Examining the contribution of macrophage subtypes in CNV formation, Zandi et al.⁴⁹ observed that the M1 macrophage transforming cytokine cocktail (INF- γ and LPS) injected into the vitreal cavity reduced CNV size and leakage. Taken together, the proinflammatory or anti-inflammatory direction of the immune response *in vivo* seems to depend on the various LPS treatment regimens (before or after CNV induction, location and dosage of injection) used in each individual study. These regimens activate different signaling pathways determining the responsiveness and polarization of microglia/macrophages and thus explaining the variability of experimental outcome. However, all of these studies underline the immune modulatory influence of LPS on changes associated with AMD.

Although our prospective study provides new insights into the association between incidence of AMD and a proxy of plasma LPS burden, limitations of this study have to be acknowledged.

Because to date no standardization of LPS quantification exists, and the widely used limulus amoebocyte lysate assay only measures the reactive fraction of LPS, we applied a proxy method to estimate the LPS exposure by translating esterified 3-OH FAs assessed by LC-tandem MS into LPS exposure.^{38,50} However, in certain circumstances esterified 3-OH FA in plasma can also indicate the presence of lipid A without acyloxyacyl structures, which are essential for bioactive signaling.¹⁵ Thus the analysis of 3-OH FAs may have led to misclassification of the exposure

itself, because LPS exposure may be different from 3-OH FAs. Furthermore, the assessment of exposure was based on a single blood draw, performed about eight years before the first ophthalmological examination. However, differential misclassification of 3-OH FAs in AMD and non-AMD cases seems unlikely, and thus misclassification of LPS exposure would tend to bias the associations with AMD toward the null.

Regarding potential confounders, we cannot rule out the possibility of residual confounding by factors that have not been evaluated or are suboptimally measured. In particular, the question arises whether the association between LPS and the incidence of early AMD is merely diet related. Indeed, healthy diet patterns such as the Mediterranean diet or prudent diet have been associated with low circulating 3-OH FAs.⁴³ There is also evidence suggesting that high consumption of saturated fats increases postprandial LPS concentrations.^{51,52} However, our results were similar in the unadjusted and the fully adjusted model (which includes adjustment for Mediterranean diet and circulating lipids), suggesting that our results were not highly confounded by nutritional status. Nevertheless, residual confounding because of measurement error (in particular, the Mediterranean diet score is based on food questionnaires relying on the subjects' memories, rendering this score prone to noise) or to other dimensions of diet that were not assessed in this study may be present.

Selection bias cannot be completely dismissed in our study, because participants included in this analysis were slightly younger and had higher LDL levels than nonparticipants.

Another limitation of our study was the small number of patients with advanced AMD, which might have induced insufficient statistical power for detecting existing associations. This may in part explain the absence of significant relationships with advanced AMD.

The strengths of the study include a large and well-defined cohort, a detailed standardized ophthalmologic examination, and long follow-up time. High-quality retinal imaging reviewed by independent retina specialists ensured negligible misclassification of AMD cases. Our analyses accounted for time to development of AMD in each individual eye and included a genetic risk score based on 49 single nucleotide polymorphisms associated with AMD.⁴¹ Dietary assessment was conducted according to the MEDILITE score.⁴² In addition, our findings are based on prospective follow-up, thus limiting reverse causation. However, only randomized clinical trials can prove the causal nature of the associations.

CONCLUSIONS

An association between higher plasma esterified 3-OH FAs and incident early AMD was observed in our cohort, suggesting that long-term proinflammatory exposure to LPS is involved in the early pathophysiological processes of AMD. This study adds to the available literature on LPS and AMD underlining the inflammatory component of AMD development. However, future studies are warranted to explore this complex relation and to better understand the underlying mechanisms.

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Results

3.1.3 Discussion

The results of Article 1 underscore the role of LPS in AMD in humans. We report that higher plasma levels of esterified 3-OH FAs, a proxy for LPS exposure, are linked to an increased risk of incident early AMD. This further confirms the role of inflammation in AMD development.

The immunostimulatory effects of LPS include an altered production of key inflammatory mediators, such as cytokines and chemokines. As LPS is naturally found in the human microbiota, microbial dysbiosis may lead to LPS translocation into the bloodstream.

Circulating LPS has been hypothesized to play a role in AMD pathogenesis. *In vitro* data has demonstrated LPS induced RPE-cytotoxicity, involving proinflammatory autocrine signaling and suggesting the role of LPS in AMD.²²⁷ Using purified B-cells from AMD patients and control subjects, an altered B- cell antibody production in response to LPS in AMD patients was reported, which may contribute to AMD pathogenesis.²²⁸ Depending on LPS dosage, timing, and administration methods, *in vivo* models of CNV have reported both significant reduction of CNV size with LPS treatment, as well as exacerbation of CNV. However, all studies underline the complex immune-modulatory effects of LPS on AMD.^{229–234}

Strikingly, we found an association between plasma esterified 3-OH FAs exclusively with early AMD and not advanced AMD. This might be due to the following factors or interactions of these: i) LPS triggers proinflammation only in the early pathogenic processes of AMD; ii) the time lag to the development of advanced AMD is too long. Therefore, no association between a plasma inflammation parameter measured in 1999 and advanced AMD, developing later and on the grounds of early AMD, could be found; iii) since the number of participants with incident advanced AMD is lower than for early AMD, our analysis may have been underpowered to find an association between LPS and advanced AMD.

Limitations of our study have to be taken into account, including the non-availability of standardized LPS quantification, and thus the application of a proxy method to estimate the LPS exposure by translating esterified 3-OH FAs assessed by LC-tandem MS into LPS exposure.²¹⁵ (The detailed protocol can be found as Appendix 1). LPS was measured only once, several years before the first AMD assessment. Thus, we may have underestimated the link between LPS and AMD. Also, residual confounding cannot be completely ruled out. In particular, the link between diet and LPS potentially bears confounding risk as it has been shown that healthy diet patterns are associated with low circulating 3-OH FAs,²¹⁹ and that

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high consumption of saturated fats increases postprandial LPS concentrations.^{198,199}

However, our results were similar in both the unadjusted and the fully adjusted model (which included adjustment for Mediterranean diet and circulating lipids), indicating that our results were not strongly confounded by diet and lipids. Further, residual confounding due to measurement error may be present since the Mediterranean diet score is based on self-reported food questionnaires, which are subject to recall bias. Additionally, other aspects of diet not adjusted for in this study could contribute to confounding (e.g. sugar and processed food intake, alcohol consumption, etc.). Finally, we also cannot entirely rule out selection bias, as participants in the analysis were slightly younger and had higher LDL levels than nonparticipants.

In conclusion, our study describes a link between a blood biomarker of LPS exposure and the incidence of early AMD in a cohort of elderly adults. However, more research is needed to establish causality and explore the long-term impact of LPS on retinal health. Prospective studies, with repeated assessments of both LPS exposure and AMD, are particularly important to determine whether LPS exposure could be a modifiable risk factor for AMD, potentially offering new preventive or therapeutic strategies. Since LPS is associated with metabolic conditions like obesity, insulin resistance, and cardiovascular diseases, targeting systemic inflammation and LPS exposure may not only reduce the risk of these conditions but also slow down or prevent the progression of AMD, thus promoting healthy aging. This is especially relevant given the aging population and the rising burden of AMD.

Results

3.2 Association of LPS with RNFL thickness

3.2.1 Introduction

Glaucoma comprises a group of diseases that cause irreversible loss of retinal ganglion cells, leading to progressive vision loss. Affecting over 3.5 % of adults aged 40 to 80 worldwide, glaucoma is the globally leading cause of irreversible blindness.¹¹⁶

In epidemiological research, the term glaucoma is generally reserved for individuals with confirmed, visually significant damage to the optic nerve. Following this definition, glaucoma is an optic neuropathy characterized by structural damage to the optic nerve and related functional impairment.¹¹⁷

Epidemiological research has shown that the predominant type of glaucoma in people with European ancestry, which comprises the Alienor cohort, is POAG.¹¹⁶ POAG is often asymptomatic until advanced stages, delaying diagnosis.

Recent advances in retinal imaging, however, allow to detect already early changes in the RNFL. Thinning of the RNFL is a biomarker of retinal neurodegeneration, and a hallmark of glaucoma. The noninvasive SD-OCT technology allows for fast high- resolution cross-sectional measurements of peripapillary RNFL thickness with an axial resolution of 5 μm and high reproducibility.^{20,126} Thus, the application of SD-OCT enables precise quantification of changes in the RNFL thickness and helps to identify patients at risk and at early stages of disease.

The exact mechanisms of POAG remain unclear, although inflammation and oxidative stress are implicated. Recent research highlights the role of the microbiome in glaucoma, suggesting that altered microbiota can contribute to systemic inflammation and ocular damage.^{188,235,236} Studies have shown that LPS exposure can worsen neuronal loss and inflammation in glaucoma models.^{190,191}

The aim of Article 2 was to explore the connection between 3-OH FA, a proxy of LPS, and RNFL thickness in a population-based cohort of elderly adults.

Results

3.2.2 Article 2

Association of Lipopolysaccharide-Type Endotoxins with Retinal Neurodegeneration: The Alienor Study

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Purpose: Lipopolysaccharide (LPS)-type endotoxins are naturally found in the gut microbiota and there is emerging evidence linking gut microbiota and neuroinflammation leading to retinal neurodegeneration. Thinning of the retinal nerve fiber layer (RNFL) is a biomarker of retinal neurodegeneration, and a hallmark of glaucoma, the second leading cause of blindness worldwide. We assessed the association of a blood biomarker of LPS with peripapillary RNFL thickness (RNFLT) and its longitudinal evolution up to 11 years.

Design: The Alienor study is a single center prospective population-based cohort study.

Subjects: The studied sample of this study includes 1062 eyes of 548 participants receiving ≥ 1 gradable RNFL measurement.

Methods: Plasma esterified 3-hydroxy fatty acids (3-OH FAs) were measured as a proxy of LPS burden. Retinal nerve fiber layer thickness was acquired using spectral-domain OCT imaging every 2 years from 2009 to 2020 (up to 5 visits).

Main Outcome Measures: Associations of plasma esterified 3-OH FAs with RNFLT were assessed using linear mixed models.

Results: Mean age of the included 548 participants was 82.4 ± 4.3 years and 62.6% were women. Higher plasma esterified 3-OH FAs was significantly associated with thinner RNFLT at baseline (coefficient beta = -1.42 microns for 1 standard deviation-increase in 3-OH FAs, 95% confidence interval $[-2.56; -0.28]$, $P = 0.02$). This association remained stable after multivariate adjustment for potential confounders. No statistically significant association was found between 3-OH FAs and longitudinal RNFLT change.

Conclusions: Higher plasma esterified 3-OH FAs were associated with thinner RNFLT at baseline, indicating an involvement of LPS in the early processes of optic nerve neurodegeneration and highlighting the potential importance of the human microbiota in preserving retinal health.

Financial Disclosure(s): Proprietary or commercial disclosure may be found in the Footnotes and Disclosures at the end of this article. *Ophthalmology Science* 2025;5:100610 © 2024 by the American Academy of Ophthalmology. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).



Supplemental material available at www.ophtalmologyscience.org.

Glaucoma comprises a heterogeneous group of diseases characterized by irreversible progressive loss of retinal ganglion cells (RGCs). Data from population-based surveys indicate that globally $>3.5\%$ of adults aged 40 to 80 years are affected by glaucoma, which equates to >95 million people.^{1,2}

All forms of glaucoma are characterized by RGC loss, retinal nerve fiber layer (RNFL) thinning, and cupping of the optic disc. The anatomy of the anterior chamber angle defines open-angle and angle-closure glaucoma. Glaucoma is further classified into primary or secondary, where primary cannot be contributed to any known cause and secondary glaucoma develops due to other disease or injury. Several other forms of glaucoma exist, including developmental glaucoma.

In people with European, Hispanic, and African ancestry, the most common form of glaucoma is primary open-angle glaucoma (POAG), whereas angle-closure glaucoma is more common in Asia.³ The most important risk factors (both for open-angle and angle-closure glaucoma) are age, elevated intraocular pressure, and positive family history. For open-angle glaucoma, myopia has been consistently reported as a risk factor.

As early-stage glaucoma is mostly asymptomatic, diagnosis is frequently delayed. In the last decades, spectral-domain OCT (SD-OCT) has facilitated noninvasively characterizing changes in the RNFL more distinctly, enabling quantification of progression already at a very early stage of disease by providing reproducible measurements of

peripapillary RNFL thickness (RNFLT) with an axial resolution of 5 μm .⁴

Although to date many different types of medication and surgery exist for glaucoma, their common aim is to prevent further damage as no treatment for irreversible damage to the optic nerve exists. Indeed, the multifactorial nature of POAG so far prevents the modeling of this disease in vitro and in vivo and thus the exact pathogenesis remains unclear.

Several human and animal studies indicate inflammatory changes in the RGC layer and the optic nerve head. In particular, a wide range of oxidative stress-related markers were found in glaucomatous patients, which may contribute to damage in the optic nerve. In recent years the “gut-eye” axis, a link between gut microbiota, diet, and micronutrients, has been described in ophthalmic pathologies, including glaucoma.^{5,6} Growing evidence also indicates the role of oral microbiota in glaucoma pathophysiology. Altered microbiota may impair the integrity of surface barriers allowing pathogens, bacterial endotoxins like lipopolysaccharide (LPS)-type endotoxins, cytokines, and other proinflammatory substances to pass into circulation, resulting in systemic inflammation, potential impairment of the immune response, and/or local ocular inflammation.

As component of the outer membrane of Gram-negative bacteria, LPS-type endotoxins are naturally found in the human microbiota. Mounting evidence indicates the involvement of LPS in the development of many chronic diseases, including glaucoma.⁷ In the eye, proinflammatory signaling via LPS occurs in microglia, perivascular macrophages, dendritic cells, photoreceptors, and retinal pigment epithelium cells and has been implicated in retinopathy of prematurity, diabetic retinopathy, age-related macular degeneration, and glaucoma (Fig 1). Low-dose LPS administration in glaucoma animal models resulted in enhancement of axonal degeneration and neuronal loss.⁸ Also, increased neuronal apoptosis and evidence of microglial activation after LPS exposure has been reported.⁹

Thus, this study was conducted to further investigate the role of a serum biomarker of LPS exposure with peripapillary RNFLT in the framework of a population-based cohort study of older adults.

Methods

Study Cohort

The Alienor study is a prospective population-based cohort study initiated in 2006 to assess the epidemiology of age-related eye diseases. Subjects of the Alienor study were recruited from the population-based Three City (3C) study cohort of Bordeaux (France).¹⁰ The 3C study is a multicenter population-based prospective cohort study initiated in 1999 to assess the vascular risk factors of dementia. The 3C study included community-dwelling individuals aged >65 years randomly drawn from electoral rolls. Between 1999 and 2001, 9294 subjects were recruited in 3 French cities: Bordeaux ($n = 2104$), Dijon ($n = 4931$), and Montpellier ($n = 2259$).

During the third follow-up of the 3C study (2006–2008), an ophthalmic examination was offered to all subjects of the 3C Bordeaux cohort, which marks the baseline of the Alienor study. A

total of 963 subjects were enrolled at baseline and have been examined every 2 years ophthalmically in parallel with the 3C study. In 2024, the sixth and final follow-up visit of the Alienor cohort was completed, which correlates to up to 18 years of ophthalmic follow-up (<http://www.alienor-study.com/langue-english-1.html>, accessed on July 30, 2024). More details on both the 3C study cohort and the Alienor study cohort have been already published elsewhere.^{10,11}

The Alienor study followed the tenets of the Declaration of Helsinki and was approved prospectively by the Ethical Committee of Bordeaux (Comité de Protection des Personnes Sud-Ouest et Outre-Mer III, code 2006/10) in May 2006. Informed consent of all participants of the Alienor study was obtained after explanation of the nature and possible consequences of the study.

Ophthalmic Examination and Imaging

The initial Alienor study protocol includes visual acuity, refraction, intraocular pressure, and 45° nonmydriatic high-resolution color retinal photographs (TRC NW6S).¹¹ Starting from the first follow-up visit (2009–2011), SD-OCT examinations of the macula and the optic nerve were included to the Alienor study protocol (Spectralis; Heidelberg Engineering). Thus, SD-OCT examinations are available at 5 time points from 2009 to 2020. The same OCT device was used at all follow-up visits.

For the peripapillary SD-OCT RNFL acquisition, the high-resolution protocol was employed. Retinal nerve fiber layer examinations were performed using 1536 A-scans of low coherence near-infrared (840 nm) light beam. Thickness measurements of peripapillary RNFL were calculated using the circle scan provided by the machine, which consisted of a 3.45-mm diameter circle scan centered on the optic disc. Global peripapillary RNFLT was automatically segmented and calculated (Fig S2, available at www.ophtalmologyscience.org). All images were acquired and reviewed by specially trained technicians and manual corrections were performed if necessary. Correction for fovea-disc orientation is incorporated in the software and a real-time eye tracking system is used to compensate for eye movements. Signal strength <15 decibels (range, 0–40) or acquisitions with artifacts were excluded from the analysis.¹²

Assessment of LPS-Type Endotoxins

All plasma measurements were assessed using blood samples collected during the 3C baseline visit (1999–2001).

Plasma esterified 3-hydroxy fatty acids (3-OH FAs) were quantified as a proxy of total plasma LPS burden using liquid chromatography tandem mass spectrometry. This method was previously described by Pais de Barros et al and quantifies plasma esterified 3-OH-FAs serve as a proxy for total plasma LPS burden.¹³ The detailed protocol can be found in [Supplementary Methods 1](#) (available at www.ophtalmologyscience.org).

Covariates

Sociodemographic data, lifestyle characteristics, and medical variables were collected at 3C baseline.¹¹

Medical variables including diabetes (fasting blood glucose ≥ 7 mmol/L or diabetes medication), hypertension (blood pressure $>140/90$ mm Hg or antihypertensive medication), self-reported cardiovascular history, and systemic lipid-lowering drugs were also assessed at 3C baseline. Fasting plasma lipids were measured at the Biochemistry Laboratory of the University Hospital of Dijon, France using routine enzymatic techniques.

Family history of glaucoma and axial length measurement were collected at Alienor baseline. Intraocular pressure was noted at time of first RNFL measurement.

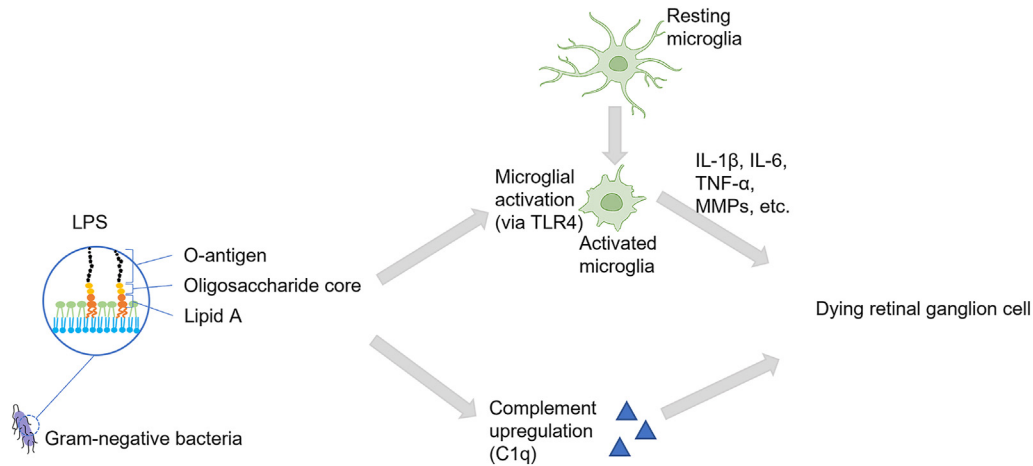


Figure 1. Potential impact of LPS on retinal ganglion cell death: impaired gut microbiota leads to translocation of proinflammatory signaling including bacterial LPS into systemic circulation. LPS induces an upregulation of TLR4 and the complement system in the retina. Microglia are among the first cells to be activated via TLR4, which in turn triggers the release of inflammatory cytokines activating apoptotic pathways leading to retinal ganglion cell death. IL-1 β = interleukin 1 β ; IL-6 = interleukin 6; LPS = lipopolysaccharide; MMPs = matrix metalloproteinases; TLR4 = Toll-like receptor 4; TNF- α = tumor necrosis factor- α .

The Mediterranean diet score is based on the MEDI-LITE score, which measures adherence to Mediterranean diet based on the literature and was developed by Sofi et al.¹⁴ This score is based on a 148-items validated food frequency questionnaire and reflects the level of adherence to the Mediterranean diet, ranging from 0 (poor adherence) to 18 (highest adherence).

The social deprivation index, a socioeconomic index based on the French European deprivation index adapted to elderly people, was calculated at the 3C baseline residential address.¹⁵

Statistical Analyses

Associations of plasma esterified 3-OH FAs with peripapillary RNFLT were estimated using linear mixed models, with the eye as the unit of analysis, as described previously.¹⁶ Interaction between 3-OH FAs and time was used to account for longitudinal effects.

All conditions for the application of the linear mixed model were verified. The linearity of quantitative variables was investigated using restricted cubic spline functions. Z score standardization was applied to the exposure variable 3-OH FAs.

Multivariate imputation by chained equations was performed to impute missing covariates, assuming that missing data were Missing At Random; more details can be found here.^{17,18} The fraction of missing/imputed data can be found in Table S1 and S2 (available at www.ophtalmologyscience.org). Multivariate adjustment was performed after imputation. Model 1 was adjusted for age and sex. Model 2 was further adjusted for the most relevant potential confounders identified from the literature and with a directed acyclic graph.

R software version 4.3.3 (R Foundation for Statistical Computing) was used for statistical analyses.

Results

Characteristics of the Studied Sample

Peripapillary RNFL examinations were included to the Alienor study protocol at the first follow-up visit. Thus, the studied sample comprises all examinations beginning with the first follow-up visit of the Alienor study. Of the

examined 888 participants, 411 eyes of 225 participants had no gradable RNFL scan (Fig 3). Another 303 eyes of 155 participants were excluded because of missing data for plasma esterified 3-OH-FA levels, leaving 1062 eyes of 548 subjects for further analyses. In total, 2497 RNFL examinations of 548 subjects were analyzed.

Among the 548 included participants, mean age at first RNFL measurement was 82.4 years (± 4.3), of which 343 (62.6%) were female (Table 3). Almost two-thirds of the study sample were never smokers, 15.3% were obese (body mass index >30 kg/m²), and the most common comorbidity was hypertension (72.3%). The average number of drugs consumed by each participant was 3.9 (± 2.6). In particular, 15.5% of the participants took anti-inflammatory medication and 33.0% lipid-lowering medication, respectively. Included participants tended to be slightly younger and were less likely to have hypertension. No significant difference was observed for other sociodemographic, lifestyle, or medical data (Table 3). A total of 180 (32.8%) participants received 1 RNFL examination, 147 (26.8%) received 2, 103 (18.8%) received 3, 64 (11.7%) received 4, and 54 (9.9%) received 5. For the 368 participants receiving >1 RNFL examination, the average follow-up time was 4.7 years (range 1.0–10.3).

Multivariate Analysis

3-Hydroxy fatty acids were standardized using the formula $Z = (x - \mu) / \sigma$, where Z is the standard score, μ the mean, and σ the standard deviation of 3-OH FAs, respectively. Measured concentration of 3-OH FAs as well as the standardized values together with a Gaussian overlay can be found in Figure 4.

Associations between esterified 3-OH FAs and RNFLT are shown in Table 4. Using linear mixed model analysis, higher exposure to esterified 3-OH FAs was significantly associated with reduced RNFLT after adjustment for age and gender (model 1: coefficient beta [β] = -1.42 microns

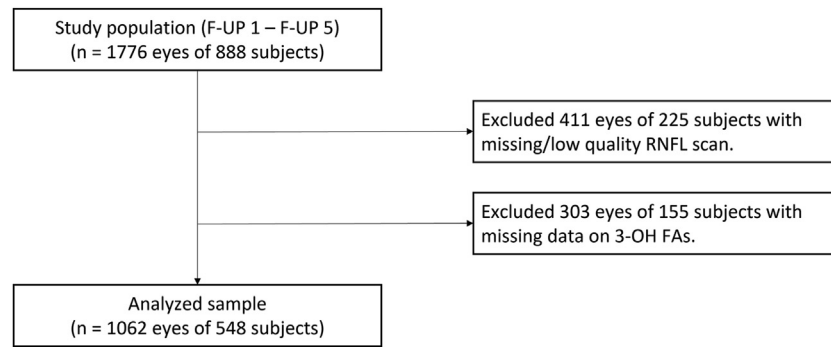


Figure 3. Flow chart showing selection of participants for analyses. Data from the Alienor study, follow-up 1 to follow-up 5; 2009 to 2020. 3-OH FAs = 3-hydroxy fatty acids; F-UP = follow-up examination; RNFL = retinal nerve fiber layer.

for 1 SD-increase in 3-OH FAs, 95% confidence interval [CI] [−2.56; −0.28], $P = 0.02$).

Further multivariate adjustment showed consistently a statistically significant association of exposure to 3-OH FAs with RNFLT (model 2: $\beta = -1.44$, CI [−2.56; −0.33], $P = 0.01$).

Mean decrease of RNFLT over time derived from both model 1 and 2 was $0.56 \pm 0.05 \mu\text{m}/\text{year}$ ($P < 0.001$). However, the longitudinal follow-up of participants with higher exposure to esterified 3-OH FAs did not show any statistical association to RNFLT change during the follow-up

Table 3. Description of the Study Population at Baseline RNFL Measurement (Mean $\pm$ SD or n [%])

Characteristics	Included (n = 548)	Nonincluded (n = 340)	P Value
	Mean ± SD or n (%)		
Age	82.4 ± 4.3	84.4 ± 5.0	<0.001
Sex			0.05
Male	205 (37.4%)	105 (30.9%)	
Female	343 (62.6%)	235 (69.1%)	
Smoking (pack-years)	n = 543	n = 332	0.13
Never smoked	352 (64.8%)	233 (70.2%)	
<20	98 (18.0%)	48 (14.5%)	
≥20	93 (17.1%)	51 (15.4%)	
BMI (kg/m ²)	n = 548	n = 331	0.84
[0, 18.5)	3 (0.5%)	5 (1.5%)	
[18.5,25)	212 (38.7%)	133 (40.2%)	
[25, 30)	249 (45.4%)	133 (40.2%)	
[30, 59]	84 (15.3%)	60 (18.1%)	
Social deprivation index	0.6 ± 2.2	0.7 ± 2.2	0.57
Plasma lipids (mmol/L)			
LDL cholesterol	3.7 ± 0.8	3.5 ± 0.8	0.08
HDL cholesterol	1.6 ± 0.4	1.6 ± 0.4	0.55
Triglycerides	1.2 ± 0.6	1.3 ± 0.7	0.27
Diabetes	40 (7.3%)	30 (8.8%)	0.12
Hypertension	396 (72.3%)	267 (78.5%)	0.03
Cardiovascular diseases	43 (7.8%)	35 (10.3%)	0.25
Mediterranean diet score	10.6 ± 2.1	10.6 ± 2.0	0.88
Lipid-lowering medication	181 (33.0%)	102 (30.0%)	0.40
Family history of glaucoma	n = 537	n = 203	
	73.0 (13.6%)	21.0 (10.3%)	0.29
Axial length	n = 321	n = 119	
Right eye	23.5 ± 1.2	23.8 ± 1.9	0.09
Left eye	23.4 ± 1.1	23.7 ± 1.7	0.16
Intraocular pression	n = 542	n = 330	
Right eye	13.4 ± 2.3	13.3 ± 2.3	0.40
Left eye	13.6 ± 2.4	13.4 ± 2.2	0.21
Global peripapillary RNFL thickness (μm)	n = 1062 eyes	-	-
	89.8 ± 14.5		

BMI = body mass index; HDL = high-density lipoprotein; LDL = low-density lipoprotein; RNFL = retinal nerve fiber layer; SD = standard deviation. Alienor study, 2009 to 2020.

Bold values represent statistically significant results.

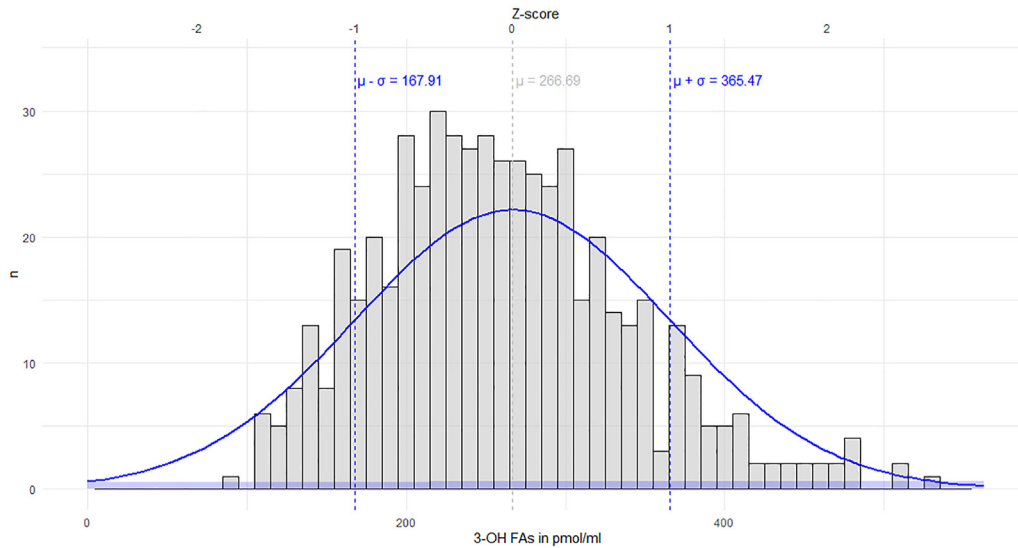


Figure 4. Distribution of measured concentration of the plasma esterified 3-OH FA with Gaussian curve overlay. 3-OH FA = 3-hydroxy fatty acid; Z-score = standard score, μ = mean, σ = standard deviation.

of the study (model 1: $\beta = 0.03$, CI $[-0.08; 0.13]$, $P = 0.60$, model 2: $\beta = 0.03$, CI $[-0.08; 0.14]$, $P = 0.58$).

The evolution of RNFLT over the follow-up predicted by model 2 is presented in Figure 5. This graphical representation of model 2 shows a clear association of higher exposure of esterified 3-OH FAs with thinner RNFLT at baseline, comparing $Z = 1$ to $Z = -1$ (i.e., a difference of 2 standard deviations in 3-OH FAs). Figure 5 also illustrates the mean decrease of RNFLT over time and reveals an almost parallel RNFLT evolution of participants with and without higher exposure to esterified 3-OH FAs, corresponding to the lack of interaction with time.

Discussion

In this cohort, higher levels of 3-OH FAs, a proxy of plasma LPS exposure, were significantly associated with thinner peripapillary RNFLT at baseline. This significant association remained stable after multivariate-adjustment for potential confounders.

To our knowledge, this is the first study analyzing the association between plasma-endotoxin exposure and RNFLT. However, emerging research suggests a potential link between alterations in human microbiota composition, leading to higher plasma-endotoxin exposure, and glaucoma.^{7,8}

Looking into human data, Collins et al found an association between POAG and mitochondrial DNA variants, which were also associated with alterations in the gut microbiome in an earlier association study of mitochondrial variation.^{19,20} Recently, differences in certain human gut microbial taxa between patients with POAG and controls have been described.^{21,22} Also a positive correlation of *Streptococcus* in fecal samples with RNFL has been reported.²¹ Recently, a Mendelian randomization analysis assessed the causal links between gut microbiota taxa and POAG.⁶ Using single-nucleotide polymorphisms associated with human gut microbiome from a multiethnic cohort of 18 340 individuals, and data for POAG including 133 492 cases and 90 939 controls, they reported that with high statistical powers of the causal

Table 4. Associations between Esterified 3-Hydroxy Fatty Acids and Peripapillary Retinal Nerve Fiber Layer Thickness

	Baseline		Longitudinal Change ($\mu\text{m}/\text{yr}$)	
	β (95% CI)	P value	β (95% CI)	P value
Model 1*	-1.42 [-2.56; -0.28]	0.02	0.03 [-0.08; 0.13]	0.60
Model 2†	-1.44 [-2.56; -0.33]	0.01	0.03 [-0.08; 0.14]	0.58

CI = confidence interval.

Alienor study, 2009 to 2020 (n = 548; 2497 examinations).

*Model 1 was adjusted for age and sex.

†Model 2 was adjusted for age, sex, social deprivation index, family history of glaucoma, axial length, intraocular pressure, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglycerides, diet quality, body mass index, and lipid-lowering medication (after imputation of missing covariates).

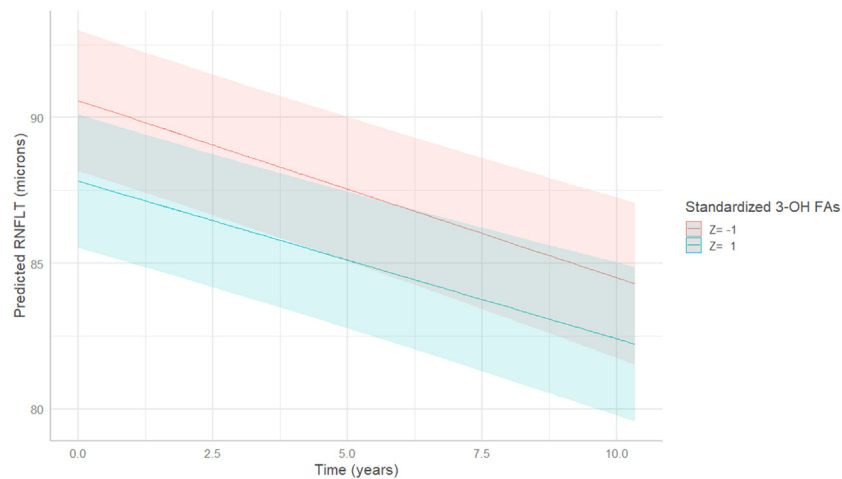


Figure 5. Longitudinal evolution of the mean RNFLT according to 3-OH FAs exposure, predicted by multivariate linear mixed model (model 2). 3-OH FAs were standardized using the formula $Z = (x - \mu) / \sigma$, where Z is the standard score, μ the mean, and σ the standard deviation of 3-OH FAs, respectively. Data from the Alienor study, follow-up 1 to follow-up 5; 2009 to 2020. 3-OH FAs = 3-hydroxy fatty acids; RNFLT = retinal nerve fiber layer thickness.

estimates (power >0.8) *Odoribacter* genus, *Ruminiclostridium9* genus, and *Eubacterium rectale* group, were associated with an increased risk of POAG.^{6,23} The authors found that intraocular pressure, central corneal thickness, and vertical cup-to-disc ratio played mediation effects between genus *Ruminiclostridium9*, genus *Alloprevotella*, phylum *Euryarchaeota*, and POAG, respectively, suggesting an impact of specific taxa on the pathogenesis of POAG.

Mechanistically, a mouse study showed that mice raised in absence of commensal microflora do not develop glaucomatous T-cell responses or associated neurodegeneration, indicating that glaucomatous neurodegeneration is mediated in part by T cells that are presensitized by exposure to commensal microflora.²⁴ Another animal study showed that reducing glaucoma-specific gut microbiota significantly reduced RGC loss by alleviating the activation of retinal microglia cells and overproduction of inflammatory cytokines.²² Furthermore, they highlighted that fecal microbiome transplantation from glaucoma patients compared with healthy subjects significantly increased retinal inflammation in mice.

The oral cavity is another important habitat of human microbiota, and dysbiosis of oral microbiome has been described to affect glaucoma progression.²⁵ In a case-control study, subjects with glaucoma showed significantly higher oral bacterial loads and fewer teeth.⁸ Using an animal model of glaucoma, low-dose LPS injected subcutaneously led to enhancement of axonal degeneration and neuronal loss and was accompanied by microglial activation in the optic nerve and retina as well as upregulation of Toll-like receptor 4 signaling and the complement system.⁸ Another case-control study by the same research group found *Streptococci* load to be higher in the saliva of patients with POAG than in controls.²⁶ Using machine learning prediction models, Yoon et al revealed significant depletion of the genus *Lactococcus*, whereas the genus *Faecalibacterium* was enriched in the oral

microbiome of patients with glaucoma.²⁷ Taken together, the data of both human and animal studies underline that the oral and gut microbiome in patients with glaucoma was different from control participants, indicating an impact of microbiome dysbiosis on the pathogenesis of glaucoma.

The strengths of this study include a large and well-defined cohort, a detailed standardized ophthalmic examination, and long follow-up time. Our results show a cross-sectional association of higher plasma esterified 3-OH FAs and RNFLT, potentially linking human microbiota with pathophysiological processes leading to optic nerve degeneration. The lack of association on the longitudinal analysis might be related to the single blood draw conducted approximately 8 years before the initial RNFL examination and to the increasing age of the cohort. Another limitation concerns the quantification of LPS: to date, no standard exists and the commonly used limulus amoebocyte lysate assay measures only the reactive portion of LPS and is not suitable for complex matrices as plasma. Here, we employed a proxy method to estimate LPS exposure by analyzing esterified 3-OH FAs through liquid chromatography tandem mass spectrometry. However, under specific conditions, esterified 3-OH FAs in plasma may indicate the presence of lipid A without acyloxyacyl structures, crucial for bioactive signaling. Therefore, the analysis of 3-OH FAs might have led to misclassification of the exposure. However, a differential misclassification of 3-OH FAs related to RNFLT is highly unlikely, thus misclassification of LPS exposure would tend to bias the associations with RNFLT toward the null. Moreover, a risk of selection bias cannot be excluded, as included and nonincluded participants differed in age and diagnosis of hypertension. However, included and nonincluded participants were comparable for all other characteristics.

Summarized, an association between higher plasma esterified 3-OH FAs and RNFLT at baseline was observed in our cohort, suggesting that LPS exposure may be involved in the pathophysiological processes of optic nerve

neurodegeneration. This study adds to the available literature on RNFLT and LPS, and more generally oral and gut microbiomes, underlining the inflammatory component of

neurodegeneration. However, future studies are warranted to explore this complex relation and to better understand the underlying mechanisms.

Footnotes and Disclosures

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Disclosures:

All authors have completed and submitted the ICMJE disclosures form.

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HUMAN SUBJECTS: Human subjects were included in this study. The study was approved prospectively by the Ethical Committee of Bordeaux (Comité de Protection des Personnes Sud-Ouest et Outre-Mer III, code 2006/10) in May 2006. All research adhered to the tenets of the Declaration of Helsinki. All participants provided informed consent.

No animal subjects were used in this study.

Author Contributions:

Conception and design: Larsen, Delcourt

Data collection: Larsen, Féart, Pais de Barros, Gayraud, Delyfer, Korobelnik, Schweitzer, Delcourt

Analysis and interpretation: Larsen, Gayraud, Delcourt

Obtained funding: Larsen, Féart, Korobelnik, Delcourt

Overall responsibility: Larsen, Delcourt

Abbreviations and Acronyms:

3C = Three City; **3-OH FA** = 3-hydroxy fatty acid; **β** = beta; **CI** = confidence interval; **LPS** = lipopolysaccharide; **POAG** = primary open-angle glaucoma; **RGC** = retinal ganglion cell; **RNFL** = retinal nerve fiber layer; **RNFLT** = retinal nerve fiber layer thickness; **SD-OCT** = spectral-domain OCT.

Keywords:

Clinical (human) or epidemiologic studies: risk factor assessment, LPS, Microbiota, Systemic inflammation.

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Results

3.2.3 Discussion

The results of Article 2 report the cross-sectional association of higher levels of plasma 3-hydroxy fatty acids with thinner baseline RNFL. This association remained significant after adjusting for potential confounders. However, no significant association was found in the longitudinal analysis. This study is the first to examine the link between plasma endotoxin exposure and RNFL thickness, aligning with emerging research that suggests a connection between altered microbiota, increased endotoxin exposure, and glaucoma.

Concerning the link between the human gut microbiome and POAG, certain gut bacterial taxa have linked to POAG risk.^{188,190,235,237,238} Animal studies further support the link between the gut microbiome and neurodegeneration and inflammation in glaucoma.^{238,239} Mice raised in absence of commensal microflora did not develop T-cell associated neurodegeneration, indicating that T cells pre-sensitized by commensal microflora-exposure mediate glaucomatous neurodegeneration.²³⁹ Reduction of glaucoma-specific gut microbiome bacteria led to significantly reduced retinal ganglion cell loss, and fecal microbiome transplantation from glaucoma patients significantly increased retinal inflammation in a mouse model.²³⁸ Further, also dysbiosis of oral microbiome has been implicated in glaucoma progression.^{190,240}

While Article 2 offers new insights into the relationship between endotoxin exposure and RNFL thickness, several limitations must be acknowledged. This includes the reliance on a single blood sample taken around eight years before the initial RNFL exam, and the lack of a standardized method for quantifying LPS. As mentioned in Article 2, the 3-OH FAs quantification using LC-tandem MS might under certain conditions indicate the presence of lipid A without acyloxyacyl structures, which are crucial for bioactive signaling, potentially leading to exposure misclassification. However, differential misclassification of 3-OH FAs in relation to RNFL thickness is highly improbable, indicating that misclassification of LPS exposure is not likely to bias the associations between 3-OH FAs and RNFL thinning.

To conclude, Article 2 reported a link between blood biomarkers of LPS exposure and thinner RNFL in a cohort of elderly adults. The findings suggest that LPS exposure may contribute to the inflammatory processes leading to retinal neurodegeneration and potentially glaucoma, warranting further investigation into the role of the human microbiome in retinal neurodegeneration.

Results

3.3 Association of LPS with macular layers thickness

3.3.1 Introduction

AMD is the leading cause of blindness in the elderly in developed countries. Major risk factors include cigarette smoking, nutritional factors, cardiovascular diseases, and genetic markers.¹

The genetic risk of AMD is associated with numerous genetic loci of small to modest effect contributing to AMD. However, Complement factor H (CFH) gene at the 1q31 susceptibility locus and ARMS2/HTRA1 gene at the 10q26 locus both showed strong and consistent associations with advanced AMD in GWAS.^{65,66,69,104} In 2016, a large GWAS by an international consortium identified 52 variants at 34 genetic loci associated with AMD at a genome-wide significance level.³ The genes at these loci are primarily involved in three biological pathways: the complement system, lipid metabolism, and extracellular matrix remodeling. Based on the results of the 2016 GWAS, a weighted polygenic risk score (PRS) was derived.⁷¹ The PRS used in Article 3 was based on this weighted risk score.

In normal aging, the retina tends to thin, primarily due to neuron loss. Progression to AMD is a process building on normal aging changes that are exacerbated by genetic and environmental factors combined with oxidative stress, low-grade inflammation and immune dysregulation. As advanced AMD often leads to irreversible vision loss, there is a need to develop biomarkers predicting AMD progression. These biomarkers could be utilized to identify patients with high risk for CNV or as clinical endpoints in intervention studies.

Chronic inflammation is a known driver in AMD, where extracellular plaques and deposits lead to a local chronic inflammatory response that exacerbates the effects of primary pathogenic stimuli.²⁴¹ Therefore, analysis of induced chronic low-grade systemic inflammation due to longstanding endotoxin-exposure is promising to uncover long-term changes in AMD, in particular in respect of morphologic changes.

Advanced imaging techniques such as SD-OCT and fundus autofluorescence have enabled detailed characterization of morphological changes during the progression of AMD. And lately, automated SD-OCT layer segmentation allows for in-depth analysis of individual macular layers in retinal diseases. Recent research has linked morphological changes in the

Results

photoreceptor layer and RPE-BM complex to AMD progression, highlighting genetic associations with photoreceptor layer thinning and RPE-BM thickening.²⁴²

However, no longitudinal study has yet examined the thickness of distinct macular layers in a population-based setting and explored their association with chronic low-grade systemic inflammation and its genetic risk of AMD.

Thus, for Article 3, I exported all the segmented layers to analyze the longitudinal morphologic changes accounting for both endotoxin-exposure and genetic risk for AMD. The associations of esterified 3-OH FAs exposure and genetic risk with longitudinal morphological changes of each retinal layer were investigated using used linear mixed models.

This third part aims to shed light into the complex interplay of morphological changes, chronic inflammation and genetic susceptibility in AMD. The results of Article 3 include the associations between macular layer thicknesses, as measured using SD-OCT, and the development of intermediate and advanced AMD, as well as their relationship with AMD polygenic risk scores (PRS). The results of the analyses of esterified 3-OH FAs exposure and longitudinal morphological changes are provided in the Results section, after Article 3.

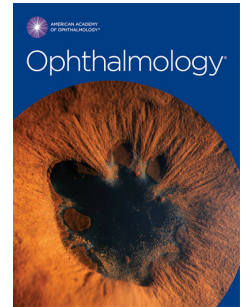
Results

3.3.2 Article

Journal Pre-proof

Neuroretinal and RPE changes and susceptibility to Age-Related Macular Degeneration: insights from the longitudinal Alienor Study

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Neuroretinal and RPE changes and susceptibility to Age-Related Macular Degeneration: insights from the longitudinal Alienor Study

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Short Title: Macular layer changes and susceptibility to AMD

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This article contains additional online-only material. The following should appear online-only: Figures S2, S4; Tables S2, S4 – S8.

Abbreviations: AMD: age-related macular degeneration, BLamDs: basal laminar deposits, BM: Bruch's membrane, CFH: complement factor H, CNV: choroidal neovascularization, ETDRS: Early Treatment Diabetes Retinopathy Study, GA: geographic atrophy, GWAS: genome-wide association study, HR: hazard ratio, GCL: ganglion cell layer, INL: inner nuclear layer, IPL: inner plexiform layer, IRL: inner retinal layers, ONL: outer nuclear layer, OPL: outer plexiform layer, ORL: retina retinal layers, PRS: polygenic risk scores, PSL: photoreceptor segment layer, RPE: retinal pigment epithelium, RNFL: retinal nerve fiber layer, SD-OCT: spectral-domain optical coherence tomography, SNP: single nucleotide polymorphism, TRL: total retinal layers.

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Abstract

Purpose: We assessed the associations of macular layer thicknesses, measured using spectral-domain OCT (SD-OCT), with incident age-related macular degeneration (AMD) and AMD polygenic risk scores (PRS).

Design: Population-based cohort study

Participants: 653 participants of the Alienor study, with biennial eye imaging from 2009 to 2024.

Methods: Macular layer thicknesses of eight distinct layers and three compound layers were automatically segmented based on SD-OCT imaging of the macula. Total and pathway specific PRS were calculated from previous AMD genome-wide association studies summary statistics. Associations of macular layer thicknesses with incident intermediate and advanced AMD were analyzed using time-dependent Cox proportional hazards models. Associations of macular layer thicknesses with PRS were assessed using linear mixed models.

Main outcome measures: Incident intermediate and advanced AMD based on fundus colour photographs and SD-OCT.

Results: Mean age at first OCT examination of the 653 participants was 82.2 ± 4.2 years and 61.3 % were women. In multivariate adjusted models, incident intermediate AMD was associated with thicker retinal pigment epithelium (RPE) - Bruch's Membrane (BM) complex in the 1 mm central circle (Hazard ratio (HR)= 1.13 for 1 μm increase; $P_{\text{FDR}} = 8.08 \times 10^{-4}$). Incident advanced AMD was associated with thicker RPE-BM complex in both the

central circle (HR= 1.09; P_{FDR} = 0.005) and the inner circle (1 mm – 3 mm) (HR= 1.28; P_{FDR} = 1.61×10^{-5}).

Over the study period, RPE-BM complex thickening in the inner circle was more pronounced in individuals with high total PRS (β = 0.06 μ m/year for 1 standard deviation increase, P_{FDR} = 1.61×10^{-10}), high complement pathway PRS (β = 0.04 μ m/year, P_{FDR} = 3.23×10^{-5}), high lipid pathway PRS (β = 0.03 μ m/year, P_{FDR} = 3.74×10^{-4}) and ARMS2 (β = 0.03 μ m/year, P_{FDR} = 0.002). Further, high total PRS and high complement-specific PRS were associated with thinner photoreceptor segment layer (PSL) at baseline and with thinning of the outer nuclear layer over the study period.

Conclusion: These results highlight the importance of RPE-BM complex thickening in the pathophysiological sequence of AMD. Further longitudinal studies are needed, in particular to determine the value of RPE-BM thickening and PSL thinning measured using SD-OCT for the clinical follow-up of AMD patients.

Introduction

Age-related macular degeneration (AMD) is a complex multi-factorial disease and the leading cause of vision loss among older adults in high income countries.^{1,2} Global pooled data of people between 45 and 85 years estimate the worldwide prevalence of AMD at 8.7%.³

AMD is classified into early, intermediate and advanced stages based on the severity of fundus lesions.⁴ Early AMD may be asymptomatic and is characterized by medium-sized drusen (63-125 μm), which are clinically visible deposits of abnormal, incompletely degraded material beneath the retinal pigment epithelium (RPE). Further progression to intermediate AMD is marked by larger drusen ($>125 \mu\text{m}$) and pigmentary abnormalities of the macula. Advanced AMD includes atrophic AMD (geographic atrophy, GA) and neovascular AMD (choroidal neovascularization, CNV), both resulting in substantial visual decline in most patients.

Over the last decades, the understanding of AMD has greatly increased. Large epidemiological studies have identified risk factors such as cigarette smoking, nutritional factors, cardiovascular diseases, and genetic markers.^{1,5} In 2016, a large genome-wide association study (GWAS) identified 52 genetic variants at 34 loci that are associated significantly with AMD. These 52 variants were used to compute a weighted polygenic risk score (PRS), which was estimated to account for more than half of the genomic heritability in the European-ancestry subjects.⁶ The genes at these 34 loci are primarily involved in three biological pathways: the complement system, lipid metabolism, and extracellular matrix (ECM) remodeling.

Furthermore, multimodal imaging modalities such as optical coherence tomography (OCT) and fundus autofluorescence have made it possible to characterize in great detail the morphological changes in the course of AMD.⁷ OCT changes in overall macular layer thickness and reflectivity are thus widely used for diagnosis and monitoring of AMD and other retinal and neurodegenerative diseases.^{8–10} However, only in recent years automated OCT layer segmentation has made it possible to study in depth the changes in distinct macular layers in retinal disease.^{11–13}

In normal aging, an overall thinning of the retina is observed, in particular due to loss of neurons.¹⁴ Further, the aging RPE undergoes structural changes, including loss of melanin granules, increase in the number of residual bodies and accumulation of the age pigment lipofuscin.¹⁵ It is thought that these changes lead to reduced antioxidant capacity of the RPE and progressive accumulation of basal laminar deposits (BLamDs) and formation of drusen.^{16,17} BLamDs are histologically found between the RPE plasma membrane and its basal lamina and would thus be measured as part of the RPE layer in automated OCT layer segmentation.¹⁸ Even though continuous enlarging BLamDs may indicate a strong risk for AMD progression and are accompanied by poor visual acuity and RPE dysmorphia, eyes with early BLamDs may show normal fundus appearance and visual acuity.¹⁶

Combined histopathological and functional studies have also evidenced that photoreceptor degeneration and loss, with a preferential vulnerability of rods, occur before disease progression to advanced AMD.^{19–21}

Recent research showed that morphologic changes within the photoreceptors and RPE-Bruch's Membrane (BM) complex may be linked to AMD and its progression. There is also

some evidence of thinner photoreceptor segment layer (PSL) and thicker RPE-BM in individuals with high genetical susceptibility to AMD²²⁻²⁵. However, to date, there has been no longitudinal analysis of the different macular layer thicknesses in a population-based study in relation to AMD.

Thus, we analyzed the longitudinal associations of macular layer thicknesses, measured using spectral-domain OCT (SD-OCT) with incident intermediate and advanced AMD and AMD PRS in a population-based cohort of older adults.

Methods

Study cohort

The Alienor study is a prospective population-based cohort study. A total of 963 participants were recruited from 2006 through 2008 from the Bordeaux cohort of the Three City (3C) study. The 3C study was initiated in 1999 and recruited individuals aged 65 years or older from electoral registers. Both the Alienor study cohort and the 3C study cohort have been described in detail elsewhere.^{5,26}

The Alienor study consists of biennial eye examinations at the Department of Ophthalmology of the University Hospital of Bordeaux between 2006 and 2024 (<http://www.alienor-study.com/langue-english-1.html>, accessed on 19 September 2024).

The Alienor study followed the tenets of the Declaration of Helsinki and was approved prospectively by the Ethical Committee of Bordeaux (Comité de Protection des Personnes Sud-Ouest et Outre-Mer III, code 2006/10) in May 2006. Informed consent of all participants of the Alienor study was obtained after explanation of the nature and possible consequences of the study.

Ophthalmic examination, retinal imaging and macular layer segmentation

The initial Alienor study protocol includes visual acuity, refraction, intraocular pressure and 45° high-resolution color fundus photographs (TRC NW6S; Topcon, Japan). Starting from the first follow-up visit (2009-2011), SD-OCT examinations of the macula and the optic nerve were included to the Alienor study protocol (Spectralis; Heidelberg Engineering, Germany). The same OCT device was used at all follow-up visits.

For the OCT acquisitions, a standardized imaging protocol was employed. All OCT assessments were performed by the same experienced technician. The Spectralis® OCT provides an automatic real-time function that adjusts for eye movement and increases image quality. For the macular cube acquisition, the following conditions were used: resolution mode, high speed; scan angle, 20°; number of B-scans, 19; pattern size, 20° × 15°; centered on the fovea and distance between B-scans, 236 µm. Single horizontal and vertical B-scan images (1536 A-scans) centered on the fovea were also performed.

The OCT-layer segmentation was performed using the inbuilt automated segmentation algorithm of the Heidelberg Eye Explorer (HEYEX 2) on the macular volume scan. The HEYEX segmentation algorithm delineates the following macular layers (**Figure 1**): retinal nerve fiber layer (RNFL), ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL), and retinal pigment epithelium (RPE). With modern SD-OCT devices, the single band previously attributed to the RPE can now occasionally be seen as 2 distinctive hyperreflective bands separated by a hyporeflective zone, particularly when pathology is present. We thus used the term RPE-BM complex instead of RPE, consistent with recent literature.²⁷ Furthermore, the layers from RNFL to ONL are combined to inner retinal layers (IRL), the photoreceptors and the RPE-BM complex to outer retinal layers (ORL), and all layers to total retinal layers (TRL). An additional layer distinct to the above listed can be calculated by subtraction of the RPE-BM complex from the ORL. This layer represents the photoreceptor inner and outer segments including the interdigitation zone and we will refer to it as photoreceptor segment layer (PSL) in the following. For each layer, the device reports the average thickness (in µm) in nine sectors (center, superior inner, temporal

inner, inferior inner, nasal inner, superior outer, temporal outer, inferior outer and nasal outer) of the 6 mm diameter Early Treatment Diabetes Retinopathy Study (ETDRS) grid centered on the fovea. As a recent cross-sectional study showed associations between macular layer thicknesses and early AMD mainly in the central (1 mm in diameter) and inner circle (consisting of the superior inner, temporal inner, inferior inner and nasal inner sector, 1–3 mm) of the ETDRS grid,²⁴ we focused our analysis on those two circles. In addition, our macular cube is too small to assess the outer ETDRS circle. The mean thickness of the inner circle was determined by calculating the average of the four inner sectors: superior inner, temporal inner, inferior inner, nasal inner.

The accuracy and reproducibility of the inbuilt automated segmentation algorithm of the HEYEX has been previously reported.²⁸

All available OCT data of the Alienor cohort were exported between January and April 2024. OCT images of participants with missing OCT data, retinal diseases and identified outliers as well as signal strength lower than 15 dB were ophthalmologically reviewed for segmentation quality. Acquisitions with artefacts and segmentation errors were excluded from the analysis.

Classification of AMD

For fundus photography, the AMD grading scheme followed the International Classification and Grading System by Bird et al., modified for drusen size, location and area according to the Multi-Ethnic Study of Atherosclerosis.^{29,30} Grading was performed from color fundus photographs in duplicate by two trained graders and inconsistencies between the two graders were adjudicated by a retina specialist.⁵ In addition, SD-OCT macular scans were interpreted for signs of advanced atrophic AMD and neovascular

AMD. Finally, classification of advanced AMD was performed by retina specialists, using all available information (ophthalmological history and treatments, color fundus photographs, SD-OCT scans).

To increase the comparability of this study with clinical routine data, in the present study, we used the Beckman AMD classification,⁴ with intermediate AMD defined as presence of large drusen and/or pigmentary abnormalities on fundus photographs, in the absence of advanced AMD (atrophic and or neovascular).

Incidence of intermediate AMD was defined as the eye progressing from no or early AMD at baseline OCT examination (2009–2010) to intermediate AMD at any time-point during the study period (2009–2020). Incidence of advanced AMD was defined as the eye progressing from no, early AMD or intermediate at baseline OCT examination (2009–2010) to advanced AMD at any time-point during the study period (2009–2020). The date of occurrence of intermediate/advanced AMD was calculated as the midpoint of the interval between the last visit without intermediate /advanced AMD and the first visit with intermediate /advanced AMD.

Covariates

Polygenic risk score (PRS): Genotyping was performed by the French Centre National de Génomique at 3C study baseline (1999–2001) using Illumina Human610-Quad BeadChips.³¹ The present PRS is based on the weighted risk score published by Fritsche and colleagues and was described in detailed elsewhere.^{6,32–34} The score corresponds to the sum of the corresponding betas multiplied by the number of minor alleles for each single nucleotide polymorphism (SNP). The betas used are calculated from the fully conditioned odds ratios of Fritsche et al. Due to the high number of missing data for the

three following SNPs: TRPM3 rs71507014, CNN2 rs67538026, and MMP9 rs142450006, these SNPs have been excluded from the risk score calculation. The present total PRS is thus based on 49 SNPs and was calculated for all participants who had available data for at least the five majors AMD-related genes (CFH rs10922109, CFH rs570618, C2 rs11603772, C3 rs2230199, and ARMS2 rs3750846).^{32,34} The pathway-specific PRS were calculated for the three main biological pathways: C2, C3, C9, CFH, CFI and TMEM97 for the complement pathway, ADAMTS9, COL4A3, COL8A1, SYN3 and VEGFA for the ECM pathway and ABCA1, APOE, CETP, LIPC for the lipid pathway.³⁴ The PRS were used as quantitative variables after Z score standardization.

Sociodemographic data, lifestyle characteristics and medical variables were collected at 3C baseline.²⁶

Medical variables including diabetes (fasting blood glucose ≥ 7 mmol/L and/or diabetes medication), hypertension (blood pressure $>140/90$ mm Hg and/or antihypertensive medication), and systemic lipid-lowering medications were also assessed at 3C baseline. Fasting plasma lipids were measured at the Biochemistry Laboratory of the University Hospital of Dijon, France using routine enzymatic techniques. Plasma esterified 3-hydroxy fatty acids (3-OH FAs) were quantified as a proxy of total plasma LPS burden using liquid chromatography tandem mass spectrometry (LC-tandem MS).³⁵ All plasma measurements were assessed using blood samples collected during the 3C baseline visit (1999–2001).

The Mediterranean diet score is based on MEDI-LITE score developed by Sofi et al.³⁶ This score is based on a 148-items validated food frequency questionnaire and reflects

the level of adherence to the Mediterranean diet, ranging from 0 (poor adherence) to 18 (highest adherence).

Statistical analyses

Cox proportional hazards model:

Associations of each macular layer thickness with incident intermediate / advanced AMD were estimated using Cox proportional hazards models. The individual eye was used as the unit of analysis. To take into account intra-individual correlation between eyes of one participant, the “cluster” term was included in the using Cox proportional hazards models in R (R Foundation for Statistical Computing, Vienna, Austria).

Time-dependent Cox proportional hazards model were used as an extension of the standard Cox model, allowing to include the repeated OCT measurements of macular layer thickness data.

Cox proportional hazards model was not performed for incident early AMD due to insufficient power, as 23.2 % (n = 223) of the cohort already had early AMD at baseline of this study.

All conditions for the application of the Cox model were verified. Linearity was checked using penalized splines with four degrees of freedom (pspline function in the Coxph function of R). The proportional hazard assumption was checked using Schoenfeld residuals.

Multivariate adjustments:

For the Cox proportional hazards model, we used two different sets of adjustments: Model 1 was adjusted for age at baseline and sex. Model 2 was further adjusted for the most

relevant potential confounders identified from the literature and with a directed acyclic graph (DAG). As some data on these variables were missing, we used multivariate imputation by chained equations (MICE) assuming that missing data were Missing At Random (MAR). Model 2 was thus adjusted after multivariate imputation.

Linear mixed model:

To study the evolution of each distinct macular layer thickness over the study period, we used linear mixed models (LMM), with the eye as the unit of analysis, as described previously.^{37,38} One model was fitted per layer and the outcome variable of each model was the macular layer thickness by circle (central or inner). The longitudinal relationship of each macular layer thickness with the fixed effects age at baseline, sex and follow-up time was studied. Interactions of age at baseline with follow-up time and sex with follow-up time were not used, as the Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC) were favorable of the less complex models.

To study the longitudinal evolution of each macular layer according to the genetic risk of AMD, the fixed effects age, follow-up time and sex, as well as PRS and the interactions between PRS and follow-up time were included in LMMs.

Finally, three sensitivity analysis were performed using LMMs, the first also included AMD stage at baseline (no/early/intermediate/advanced) as fixed effect, the second excluded all participants with any AMD stage at baseline and the third excluded all participants with intermediate or advanced AMD stage at baseline.

All conditions for the application of the linear mixed model were verified. The linearity of quantitative variables was investigated using restricted cubic spline functions.

Correction for multiple testing

To address multiple testing, the Benjamini-Hochberg procedure was applied for false discovery rate (FDR) correction.³⁹ The Benjamini-Hochberg procedure adjusts p-values in a stepwise manner aiming to limit the proportion of false positives: After p-values are assigned ranks according to their ascending value, adjusted p-values were calculated using the following formula, where:

$$p_{adjusted(i)} = \min \left(1, \min_{k \geq i} \left(\frac{m}{k} \cdot p_{(k)} \right) \right)$$

- adjusted p(i) represents the adjusted p-value for the i-th ranked p-value,
- p(k) represents the k-th ranked p-value and
- m represents the total number of hypotheses being tested.

Statistical significance was determined using an FDR-corrected P value (P FDR) of less than 0.05.

R software version 4.3.3 (R Foundation for Statistical Computing, Vienna, Austria) was used for all statistical analyses.

Results

Characteristics of the studied sample

OCT examinations were included to the Alienor study protocol from 2009. Thus, the studied sample comprises all participants of the Alienor study receiving at least one OCT examinations between the first follow-up visit (2009-2010) and the last follow up visit in February 2024. A total of 3532 OCT scans of 721 subjects were exported. Of these 344 OCT scans of 94 subjects were excluded due to low quality. Further 408 OCT scans of 174 subjects were excluded due to errors of the automatic segmentation (**Figure S2**). Thus, in total 2780 OCT examinations of 653 subjects were analyzed.

Among the 653 included participants, mean age at first OCT examination was 82.2 years (± 4.2) and 400 (61.3%) were female (**Table 1**). Approximately two-thirds had never smoked and 15.8 % were obese (Body mass index, BMI > 30 kg/m²). Prevalent hypertension was reported in 473 (72.4 %) of the participants and prevalent diabetes mellitus in 46 (7.0 %). A total of 236 (36.1 %) participants received one OCT examination, 166 (25.4 %) received two, 109 (16.7 %) received three, 82 (12.6 %) received four and 60 (9.2 %) received at least five. For the 417 participants receiving more than one OCT examination, the average follow-up time was 4.8 years (range 0.3 to 14.1).

Measurements of Macular Layer Thicknesses

The thickness of eight separate macular layers and three compound layer complexes was measured in micrometers, including the RNFL, GCL, IPL, INL, OPL, ONL, PSL, RPE-BM complex, IRL complex and ORL complex and TRL complex. The mean macular layer

thicknesses of the 1 mm central and the 1 mm - 3 mm inner ETDRS circle of these layers and layer complexes at baseline OCT examination can be found in **Table S2**.

Macular layer thicknesses and the risk of incident intermediate and advanced AMD

We analyzed the associations of macular layer thicknesses with incident intermediate and advanced AMD. To take into account the data of all available OCT follow-up examinations, we used time-dependent proportional hazard Cox Models (**Table 3**).

Incident intermediate AMD: After adjustment for age and sex, we observed an association of incident intermediate AMD with thicker RPE-BM complex in the 1 mm central circle (Hazard ratio (HR) = 1.12 for 1 μ m increase; 95% confidence interval (CI), [1.08; 1.18]; $P_{FDR} = 9.75 \times 10^{-4}$, **Table 3**). The association was similar after adjustment for AMD stage at baseline, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, triglycerides (TG), diet quality, body mass index (BMI), lipid-lowering medication, physical activity, 3-hydroxy fatty acids as a proxy of Lipopolysaccharide -type endotoxins, smoking and PRS (**Table 3**). No significant association was observed of incident intermediate AMD with any other macular layer, neither in the central circle, nor in the inner circle.

Incident advanced AMD: After adjustment for age and sex, the time-dependent Cox Model showed that thicker RPE-BM complex in the 1 mm central circle was associated with higher risk of advanced AMD (HR = 1.16 for 1 μ m increase; 95% CI, [1.10; 1.22]; $P_{FDR} = 1.01 \times 10^{-7}$) (**Table 3**). The association was similar in the fully-adjusted model (**Table 3**). Also, in the 1 – 3 mm inner circle, thicker RPE-BM complex was strongly associated with higher risk of advanced AMD in the age and sex adjusted model (HR = 1.42 for 1 μ m

increase; 95% CI, [1.30; 1.56]; $P_{FDR} = 7.49 \times 10^{-13}$), as well as in the fully-adjusted model (HR = 1.28 for 1 μm increase; 95% CI, [1.16; 1.42]; $P_{FDR} = 1.61 \times 10^{-5}$, **Table 3, Figure 3**). This corresponds to a multiplication of the risk of advanced AMD by 3.43 for an RPE-BM complex of 15 μm and by 11.8 for an RPE-BM complex of 20 μm , in comparison with an RPE-BM complex of 10 μm , in the fully-adjusted model. (**Figure 3**). We observed no statistically significant associations with other retinal layers.

Evolution of macular layers with age, sex and follow-up time

Using linear mixed model analysis, we analyzed the evolution of each macular layer in depth, first looking into the associations with age, sex and time (**Table S4, Figure S4**).

In the 1 mm central circle, the four innermost macular layers differed significantly at baseline according to sex, with female sex being significantly associated with thinner RNFL (Coefficient Beta (β) = -1.13 μm for female sex; 95% CI, [-1.53; -0.73]; $P_{FDR} = 1.79 \times 10^{-7}$), GCL (β = -1.62 μm for female sex; 95% CI, [-2.34; -0.90]; $P_{FDR} = 3.33 \times 10^{-5}$), IPL (β = -1.75 μm for female sex; 95% CI, [-2.33; -1.17]; $P_{FDR} = 3.12 \times 10^{-8}$) and INL (β = -3.90 μm for female sex; 95% CI, [-4.83; -2.97]; $P_{FDR} = 8.88 \times 10^{-15}$). Older age at baseline was significantly associated with thinner PSL at baseline (β = -0.15 μm for 1 year of age increase; 95% CI, [-0.20; -0.09]; $P_{FDR} = 1.32 \times 10^{-6}$).

In the 1 – 3 mm inner circle, older age at baseline was associated with thinner GCL (β = -0.31 μm for 1 year of age increase; 95% CI, [-0.43; -0.19]; $P_{FDR} = 1.47 \times 10^{-6}$) and thinner IPL (β = -0.20 μm for 1 year of age increase; 95% CI, [-0.27; -0.13]; $P_{FDR} = 1.79 \times 10^{-7}$) at baseline. Again, the female sex was significantly associated with thinner INL (β = -1.20 μm for female sex; 95% CI, [-1.73; -0.68]; $P_{FDR} = 2.67 \times 10^{-5}$) and also with

thinner ONL ($\beta = -3.37 \mu\text{m}$ for female sex; 95% CI, $-4.70; -2.03$]; $P_{\text{FDR}} = 3.23 \times 10^{-6}$) at baseline.

Macular layer thicknesses in the 1 – 3 mm inner circle all decreased significantly with follow-up time, except for RNFL which increased. In the 1 mm central circle, INL and RPE-BM complex increased with follow-up time, while ONL and PSL decreased.

AMD polygenic risk score (PRS) and macular layers

Using linear mixed model analysis, we then analyzed the association of each layer according to their genetic risk using the PRS for AMD, adjusting for age at baseline and sex (**Table S5**).

The AMD PRS was significantly associated with thinner PSL at baseline in the 1 mm central circle ($\beta = -0.63 \mu\text{m}$ for 1 standard deviation (SD)-increase of the PRS, 95% CI, $[-0.86; -0.40]$, $P_{\text{FDR}} = 2.42 \times 10^{-6}$, **Table S5, Figure 5**) and in the 1 – 3 mm inner circle ($\beta = -0.39$, 95% CI $[-0.52; -0.26]$, $P_{\text{FDR}} = 2.46 \times 10^{-7}$, **Table S5, Figure 5**), but was not significantly associated with the evolution of PSL with follow-up time.

The AMD PRS was associated with a small but significant ONL thinning ($\beta = -0.11 \mu\text{m}/\text{year}$ for 1 SD-increase of the PRS, 95% CI, $[-0.16; -0.06]$, $P_{\text{FDR}} = 3.08 \times 10^{-4}$, **Table S5**) and RPE-BM complex thickening ($\beta = 0.06 \mu\text{m}/\text{year}$ for 1 SD-increase of the PRS, 95% CI, $[0.04; 0.07]$, $P_{\text{FDR}} = 1.61 \times 10^{-10}$, **Table S5**) in the 1 – 3 mm inner circle. As shown in **Figure 6**, ONL was not different at baseline in participants with high (standardized PRS = +1) or low (standardized PRS = -1) genetic susceptibility, but decreased more rapidly in those with high genetic susceptibility. Regarding the RPE-BM complex, again there was no significant difference at baseline between participants with high and low PRS, but RPE-

BM complex increased with time in those with high PRS, while it decreased in those with low PRS.

Of note, other layers were not associated with AMD PRS, either at baseline or during follow-up.

Three sensitivity analyses which showed similar results i) after inclusion of AMD stage (no/early/intermediate/advanced) at baseline as fixed effect, ii) after exclusion of all participants with any AMD at baseline and iii) after exclusion of all participants with intermediate and advanced AMD at baseline (**Table S6, S7 and S8**).

Pathway-specific polygenic risk scores (PRS) and macular layers

Using linear mixed model analysis, we analyzed the genetic risk profile in more detail by looking into the association of the pathway-specific PRS with the macular layers showing significant associations with the AMD PRS (**Table 9**).

We observed a significant association of the complement pathway with ONL thinning ($\beta = -0.16 \mu\text{m}/\text{year}$, 95% CI [-0.21; -0.11], $P_{\text{FDR}} = 2.46 \times 10^{-7}$) in the 1 -3 mm inner circle, while ARMS2 was associated with ONL thickening in both the 1 mm central ($\beta = 0.12 \mu\text{m}/\text{year}$, 95% CI [0.03; 0.21], $P_{\text{FDR}} = 0.04$) and the 1 -3 mm inner circle ($\beta = 0.09 \mu\text{m}/\text{year}$, 95% CI [0.03; 0.15], $P_{\text{FDR}} = 0.02$, **Table 9**).

We also observed a significant association of the complement pathway with thinner baseline PSL in the 1 mm central circle ($\beta = -0.65 \mu\text{m}$, 95 % CI [-0.93; -0.38], $P_{\text{FDR}} = 4.72 \times 10^{-5}$) and in the 1 – 3 mm inner circle ($\beta = -0.40 \mu\text{m}$, 95 % CI [-0.56; -0.25], $P_{\text{FDR}} = 6.66 \times 10^{-6}$). However, PSL decreased less rapidly with time in those with high complement

365 pathway PRS, both in the central ($\beta = 0.07 \mu\text{m} / \text{year}$, 95 % CI [0.03; 0.11], $P_{\text{FDR}} = 3.31 \times$
366 10^{-3}) and the inner circle ($\beta = 0.03 \mu\text{m} / \text{year}$, 95 % CI [0.01; 0.05], $P_{\text{FDR}} = 0.01$, **Table 9**).
367 We did not observe significant associations of PSL with the other pathway-specific PRS.

368 Finally, we observed a significant association of the complement pathway ($\beta = 0.04 \mu\text{m} /$
369 year , 95 % CI [0.02; 0.06], $P_{\text{FDR}} = 3.23 \times 10^{-5}$), the lipid pathway ($\beta = 0.03 \mu\text{m} / \text{year}$, 95
370 % CI [0.02; 0.05], $P_{\text{FDR}} = 3.74 \times 10^{-4}$) and ARMS2 ($\beta = 0.03 \mu\text{m} / \text{year}$, 95 % CI [0.02;
371 0.05], $P_{\text{FDR}} = 0.002$) with RPE-BM complex thickening.

Discussion

This study evidenced a strong association between thicker foveal RPE-BM complex and both incident intermediate and advanced AMD. Longitudinal RPE-BM complex thickening was found to be strongly associated with total AMD PRS, and specifically with the complement, lipid metabolism and ARMS2 pathways PRS. Further, both the total AMD PRS and the complement-specific PRS were associated with thinner PSL at baseline and with longitudinal ONL thinning. Overall, our data contribute to and highlight the following results in the light of known pathophysiological mechanisms and recent literature.

First, RPE-BM complex thickening is a hallmark of AMD progression. In aging RPE cells, cumulative oxidative damage contributes to anatomical and physiological changes, including the accumulation of byproducts of the visual cycle leading to the accumulation of drusen (between the RPE basal lamina and Bruch's membrane).⁴⁰ Distinct of drusen, BLamDs, found between the RPE plasma membrane and its basal lamina, also represent early pathogenic changes in AMD.¹⁶ Thus thickening of the RPE-BM complex measured using OCT possibly include drusen, BLamDs, accumulation of lipofuscin, increase in the density of residual bodies and diffuse thickening of Bruch's membrane.¹⁴

In 2019, a cross-sectional analysis of the German population-based AugUR Study also observed RPE-BM complex thickening and PSL thinning in early AMD.²⁴ Similarly, the cross-sectional Portuguese Coimbra study showed that RPE-BM complex thickening and PSL thinning is found in more severe early AMD.²⁵ Here, we show that RPE-BM complex is associated with future development of intermediate or advanced AMD, independently of potential confounders, including AMD stage at baseline.

Schmidt-Erfurth et al. used a machine learning–based predictive model assessing the risk of conversion to advanced AMD and described the reduction of neurosensory structures, including the PSL, as an important determinant in the progression to GA.⁴¹ Further, they reported RPE-drusen complex thickening as a prominent predictor for the development of CNV. In the present study, however, we could not distinguish atrophic and neovascular AMD, due to the low number of incident advanced AMD cases.

A recent study by Zekavat et al., underlined the significance of early changes in the PSL, using the data of the UK Biobank with a broad AMD classification and including patients of an age span from 40 to 70 years.²² They reported that PSL thinning and RPE-BM complex thickening were both significantly associated with increased AMD prevalence and incidence. Interestingly, they observed accelerated PSL thinning starting at around 45 years of age, 12 years before RPE-BM complex thickening, which highlights the potential pathophysiological sequence in morphological changes in the development of AMD.²² This potential chronological sequence is in line with our finding of a highly significant association of RPE-BM complex thickening with incident intermediate and advanced AMD and could also explain the lack of finding PSL thinning, as this thinning might have occurred before the baseline OCT examination of our study cohort of participants aged 75 years or above at baseline.

Second, using repeated OCT measurements of the same individuals, we are the first to report the longitudinal evolution of each distinct macular layer according to age and gender in a cohort of people above the age of 75 at baseline. Using this data, we could show distinct sex-related differences, e.g. thinner inner macular layers in women which is in agreement with previous cross-sectional data.^{28,42}

Third, our results showed that the total PRS based on 49 of the 52 genetic variants associated with AMD at a genome-wide significance level for AMD⁶ was associated with thinner PSL at baseline. The total PRS was not associated with thicker RPE-BM complex at baseline, but with longitudinal RPE-BM complex thickening, which is again in line with the hypothesized pathophysiological chronological sequence of OCT-related changes by Zekavat et al.²² Further, we also found an association between the total AMD PRS and longitudinal ONL thinning. This seems consistent with the cross-sectional data of Brandl et al. and Farinha et al. showing that ONL is decreased in participants with severe early AMD.^{24,25}

Fourth, our results pinpoint specific mechanisms in PRS associated macular layer changes. The above used total PRS was grouped into four main biological pathways by an international consortium.³⁴ the complement system, lipid metabolism, ECM remodeling and ARMS2. Looking into those pathway specific PRS, the complement pathway was strongly associated with thinner PLS at baseline as well as longitudinal ONL thinning and RPE-BM complex thickening. Even though the genetic risk of AMD is considered to be linked to multiple genetic loci of small to modest effect, one of two major SNPs linked to advanced AMD is a variant in the complement factor H gene (CFH).⁴³⁻⁴⁶ This variant affects the protein's ability to regulate inflammation, leading to increased susceptibility to chronic inflammation and damage in the retina. Associations with factor B and complement component C2 genes further emphasized the critical role of the complement system in the development of AMD.⁴⁷ Furthermore, systemic measurements of complement activation products like C3a, C3d, and C5a have consistently shown elevated levels in AMD.⁴⁸ However, using total macular thickness OCT data, a recent GWAS by

Gao et al. did not find an association between variants in the CFH gene within the outermost circle of the ETDRS grid.⁴⁹ By contrast, another study by Zouache et al. found that variants in the CFH gene were associated with perifoveal changes in macular thickness on OCT.⁵⁰ Finally, the study by Zekavat et al., reported the variants CFH:rs570618-T, CHF:10922109-C, CFH:rs187328863-T and ARMS2:rs3750846-C to be significantly associated with PSL thinning.²²

Looking into the pathway specific PRS and longitudinal RPE-BM complex thickening reveals that multiple players are at game, of which the complement and lipid pathways seem to be strongest associated. However, in RPE-BM complex thickening, the second major AMD SNP, ARMS2, is also involved. Variants in this region are known to be associated with changes in mitochondrial function and increased inflammation, both contributing to retinal damage.^{51–53}

Taken together, the current knowledge on morphologic and functional data indicates the importance of primarily RPE-BM complex thickening and photoreceptor degeneration/loss in the development of AMD. Further, similar to the normalized database of retinal nerve fiber measurements as used in glaucoma clinics, the use of RPE-BM complex measurements could be considered in AMD clinics. These measurements are readily available in the current version of the Heidelberg Spectralis. Longitudinal change of the RPE-BM complex might serve as an indicator of patients with high risk of conversion to advanced AMD.

The strengths of the study include a large and well-defined cohort, a detailed standardized ophthalmologic examination and long follow-up time. Although our prospective study

provides new insights into the genetic and morphologic correlation in AMD, limitations of this study have to be acknowledged: The OCT protocol performed systematically in the Alienor study employed 19 B-scans with an interscan distance of 236 μm , which implies that macular thickness values are based on a relatively limited number of scans. However, research on features of retinal diseases, in particular drusen volume and exudative retinal changes, has demonstrated that less dense scans (up to an interscan distance of 240 μm) do not significantly affect drusen volume measurements or impair the detection of exudative retinal changes.^{8,54,55}

While the Heyex system's inbuilt segmentation algorithm is easily accessible, it may be less accurate than newer automated segmentation algorithms, especially when handling pathological alterations. To minimize the possibility of systematic misclassification of macular layer thicknesses, we manually reviewed all OCT scans with quality below 15dB, scans from patients with diagnosed retinal disease and any outliers (identified using linear regression). To ensure reproducibility, no manual corrections were performed, and all OCT scans with segmentations errors or disrupted retinal structure due to underlying disease were systematically excluded. Segmentation errors included missing retinal layers, overlapping or merging layers, incorrect retinal layer boundary detection, segmentation gaps, erroneous inclusion of pathological features in retinal layer. However, the measurement errors due to the acquisition protocol and the segmentation algorithm are most probably not differential and therefore would tend to attenuate the estimated associations. Future studies should strive to minimize such methodological limitations by using more numerous scans and more precise algorithms.

The genetic risk score used in this study is based on the foundational work by Fritsche et al. 2016, which identified key AMD risk SNPs.⁶ However, due to the rapid advancements in genetic research, more recent studies have significantly expanded on this by providing higher SNP resolution, particularly through fine-mapping techniques. These advancements have enabled the identification of more specific, potentially causal variants at known risk loci, especially with the inclusion of multi-ethnic cohorts.^{56,57} While these recent papers offer enhanced resolution, considering the European ethnicity of participants in the Alienor study, the comprehensive coverage of major AMD risk SNPs and the comparable discriminatory power, the use of the current PRS score based on Fritsche et al. 2016 remains justified.

Finally, we considered a wide range of potential cofounders but cannot exclude that some residual confounders or sources of misclassification could explain our observed association between RPE-BM complex thickening and incident intermediate and advanced AMD.

Conclusion

We found a strong association of thicker RPE-BM complex with incident intermediate and advanced AMD in a population-based cohort of elderly individuals. Thickening of the RPE-BM complex was also more pronounced in participants with high genetic susceptibility to AMD, as well as ONL thinning and thinner PSL at baseline. These results highlight the importance of RPE-BM complex thickening in the pathophysiological sequence of AMD. Further longitudinal studies are needed, in particular to determine the value of RPE-BM

505 complex thickening and PSL thinning measured using SD-OCT for the clinical follow-up
506 of AMD patients.

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Figure Legends

Figure 1. Example of an auto- segmented spectral domain-OCT scan using the inbuilt automated segmentation of the Heidelberg Eye Explorer.

RNFL: retinal nerve fiber layer, GCL: ganglion cell layer, IPL: inner plexiform layer, INL: inner nuclear layer, OPL: outer plexiform layer, ONL: outer nuclear layer, PSL: photoreceptor segment layer, RPE-BM: retinal pigment epithelium- Bruch's Membrane complex.

Figure 3. Association between Retinal Pigment Epithelium (RPE) - Bruch's Membrane (BM) complex thickness within the 1 – 3 mm inner circle of the ETDRS grid and incident advanced AMD by eye, using time-dependent proportional hazards Cox Model, along with exemplary OCT photos of participants with an RPE - BM complex thickness of 10 μm , 15 μm and 20 μm at baseline. The model was adjusted for age, sex, high-density (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, triglycerides (TG), diet quality, body mass index (BMI), lipid-lowering medication, physical activity, 3-hydroxy fatty acids as a proxy of Lipopolysaccharide - type endotoxins, smoking status, polygenic risk score and AMD stage at baseline. AMD stage at baseline was categorized in 3 categories (no/early/intermediate). The hazard function is represented using p-spline with 4 degrees of freedom. Exemplary OCT images of participants with an RPE-BM complex thickness of 10 μm , 15 μm and 20 μm are shown. Data from the Alienor Study, 2009–2020.
HR: hazard ratio.

Figure 5. The evolution of photoreceptor segment layer (PSL) thinning over the follow-up time for both the 1 mm central and the 1 – 3 mm inner circle of the Early Treatment Diabetic Retinopathy Study (ETDRS) grid as predicted by linear mixed models (adjusted for age, sex and follow-up time) shown for the longitudinal relationship with standardized total (PRS) for AMD. The PRS was standardized using the formula $Z = (x-\mu)/\sigma$, where Z is the standard score, μ the mean and σ the standard deviation, respectively. Data from the Alienor Study, 2009–2024.

Figure 6. The evolution of the outer nuclear layer (ONL) and the retinal pigment epithelium (RPE) - Bruch's Membrane (BM) complex in the 1 – 3 mm inner circle of the Early Treatment Diabetic Retinopathy Study (ETDRS) grid over the follow-up time as predicted by linear mixed models (adjusted for age, sex and follow-up time) shown for the longitudinal relationship with standardized total polygenic risk score (PRS) for AMD. The PRS was standardized using the formula $Z = (x-\mu)/\sigma$, where Z is the standard score, μ the mean and σ the standard deviation, respectively. Data from the Alienor Study, 2009–2024.

Table 1. Baseline Characteristics of the Study Population (n = 653), Data from the Alienor Study.

Characteristics	Mean $\pm$ SD or n (%)
Age (years)	82.2 $\pm$ 4.2
Sex	
Male	253 (38.7 %)
Female	400 (61.3 %)
Smoking (pack-years)	
Never smoked	424 (64.9 %)
< 20	122 (18.7 %)
$\geq$ 20	107 (16.4 %)
Body mass index (BMI)	
[0,18.5)	3 (0.5 %)
[18.5,25)	260 (39.8 %)
[25,30)	287 (44.0 %)
[30,59]	103 (15.8 %)
Plasma lipids (mmol/L)	
LDL cholesterol	3.6 $\pm$ 0.8
HDL cholesterol	1.6 $\pm$ 0.4
Triglycerides	1.2 $\pm$ 0.6
Diabetes	46 (7.0 %)
Hypertension	473 (72.4 %)
Cardiovascular Diseases	57 (8.7 %)
Mediterranean diet score	10.6 $\pm$ 2.0

LDL: low-density lipoprotein, HDL: high-density lipoprotein;
SD: standard deviation.

Table 3. Associations between the macular layer thicknesses and incidence of intermediate and advanced AMD. Alienor study, 2009–2020.

		Intermediate AMD (n = 103)				Advanced AMD (n = 39)			
		Central circle of the ETDRS grid		Inner circle of the ETDRS grid		Central circle of the ETDRS grid		Inner circle of the ETDRS grid	
		HR (95% CI)	P _{FDR}	HR (95% CI)	P _{FDR}	HR (95% CI)	P _{FDR}	HR (95% CI)	P _{FDR}
RNFL	Model 1	1.03 [0.97; 1.10]	0.62	1.00 [0.95; 1.05]	0.99	1.09 [0.97; 1.21]	0.53	1.06 [0.98; 1.15]	0.53
	Model 2	1.04 [0.97; 1.11]	0.60	1.00 [0.95; 1.06]	0.99	1.05 [0.93; 1.19]	0.69	1.04 [0.96; 1.13]	0.60
GCL	Model 1	1.01 [0.98; 1.04]	0.83	1.02 [0.99; 1.06]	0.53	1.03 [0.97; 1.09]	0.60	1.01 [0.96; 1.06]	0.83
	Model 2	1.02 [0.98; 1.05]	0.67	1.02 [0.99; 1.05]	0.54	1.02 [0.95; 1.08]	0.83	1.02 [0.97; 1.07]	0.73
IPL	Model 1	1.01 [0.96; 1.06]	0.92	1.03 [0.98; 1.08]	0.58	1.04 [0.96; 1.12]	0.60	1.00 [0.93; 1.08]	0.99
	Model 2	1.01 [0.96; 1.07]	0.83	1.03 [0.98; 1.08]	0.60	1.02 [0.95; 1.11]	0.75	1.02 [0.95; 1.10]	0.79
INL	Model 1	1.01 [0.98; 1.03]	0.74	0.99 [0.94; 1.04]	0.89	1.04 [1.01; 1.08]	0.13	1.03 [0.95; 1.12]	0.73
	Model 2	1.01 [0.98; 1.04]	0.60	0.98 [0.93; 1.03]	0.69	1.02 [0.98; 1.07]	0.60	1.01 [0.92; 1.11]	0.95
OPL	Model 1	1.00 [0.97; 1.03]	0.99	0.97 [0.93; 1.01]	0.55	0.99 [0.94; 1.06]	0.95	1.00 [0.93; 1.08]	0.99
	Model 2	1.00 [0.98; 1.03]	0.94	0.97 [0.93; 1.02]	0.55	1.00 [0.94; 1.06]	0.99	0.99 [0.92; 1.06]	0.90
ONL	Model 1	1.01 [1.00; 1.03]	0.54	1.01 [0.99; 1.04]	0.60	0.98 [0.95; 1.01]	0.60	0.95 [0.92; 0.99]	0.06
	Model 2	1.01 [0.99; 1.03]	0.60	1.01 [0.99; 1.03]	0.60	0.98 [0.95; 1.01]	0.53	0.96 [0.93; 0.99]	0.06
PSL	Model 1	0.97 [0.93; 1.03]	0.60	0.99 [0.90; 1.09]	0.94	0.93 [0.85; 1.03]	0.53	1.17 [1.04; 1.31]	0.07
	Model 2	0.97 [0.92; 1.03]	0.62	0.99 [0.89; 1.10]	0.95	0.96 [0.89; 1.04]	0.60	1.20 [1.02; 1.40]	0.14
RPE-BM	Model 1	1.12 [1.06; 1.18]	9.75 x 10⁻⁴	1.09 [0.97; 1.22]	0.53	1.16 [1.10; 1.22]	1.01 x 10⁻⁷	1.42 [1.30; 1.56]	7.49 x 10⁻¹³
	Model 2	1.13 [1.06; 1.20]	8.08 x 10⁻⁴	1.09 [0.97; 1.23]	0.53	1.09 [1.04; 1.14]	0.005	1.28 [1.16; 1.42]	1.61 x 10⁻⁵

*For the inner circle, the thickness was calculated as the average of the nasal/temporal/superior/inferior subfields.

^aModel 1, HR was estimated using Cox proportional model adjusted for age at baseline and sex. HR for 1 μ m increase of the respective layer. ^bModel 2, HR was estimated using Cox proportional model adjusted for age, sex, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, triglycerides (TG), diet quality, body mass index (BMI), lipid-lowering medication, physical activity, 3-hydroxy fatty acids as a proxy of Lipopolysaccharide -type endotoxins, smoking, polygenic risk score and AMD stage at baseline. For intermediate AMD, AMD stage at baseline was in 2 categories (no/early), for advanced AMD, it was in 3 categories (no/early/intermediate). HR for 1 μ m increase of the respective layer. ETDRS: Early Treatment Diabetic Retinopathy Study, CI: Confidence interval, FDR: false discovery rate, HR, hazard ratio, RNFL: retinal nerve fiber layer, GCL: ganglion cell layer, IPL: inner plexiform layer, INL: inner nuclear layer, OPL: outer plexiform layer, ONL: outer nuclear layer, PSL: photoreceptor segment layer, RPE-BM: retinal pigment epithelium- Bruch's Membrane complex.

Table 9. Longitudinal change of ONL, PSL and RPE-BM complex according to pathway-specific polygenic risk scores for AMD, Alienor study, 2009–2024. (n = 2780 examinations of 653 participants)

		Central Circle of the ETDRS Grid		Inner Circle of the ETDRS Grid*	
		β [95% CI]	P_{FDR}	β [95% CI]	P_{FDR}
ONL	Complement Pathway PRS**	0.04 [-0.89; 0.97]	0.99	0.11 [-0.62; 0.83]	0.94
	Complement Pathway PRS * Time	-0.09 [-0.17; -0.01]	0.12	-0.16 [-0.21; -0.11]	2.46×10^{-7}
	Extracellular Matrix Pathway PRS***	0.22 [-0.70; 1.14]	0.88	0.11 [-0.61; 0.83]	0.94
	Extracellular Matrix Pathway PRS * Time	0.02 [-0.06; 0.10]	0.84	-0.03 [-0.08; 0.02]	0.53
	Lipid Pathway PRS****	-0.30 [-1.22; 0.62]	0.81	0.06 [-0.66; 0.78]	0.97
	Lipid Pathway PRS * Time	-0.02 [-0.10; 0.06]	0.88	-0.02 [-0.07; 0.03]	0.72
	ARMS2	-0.16 [-1.09; 0.78]	0.94	-0.16 [-0.88; 0.57]	0.91
	ARMS2 * Time	0.12 [0.03; 0.21]	0.04	0.09 [0.03; 0.15]	0.02
PSL	Complement Pathway PRS**	-0.65 [-0.93; -0.38]	4.72×10^{-5}	-0.40 [-0.56; -0.25]	6.66×10^{-6}
	Complement Pathway PRS * Time	0.07 [0.03; 0.11]	3.31×10^{-3}	0.03 [0.01; 0.05]	0.01
	Extracellular Matrix Pathway PRS***	-0.17 [-0.45; 0.11]	0.50	-0.04 [-0.19; 0.12]	0.88
	Extracellular Matrix Pathway PRS * Time	-0.02 [-0.06; 0.02]	0.53	-0.002 [-0.02; 0.02]	0.94
	Lipid Pathway PRS****	-0.18 [-0.46; 0.09]	0.45	-0.11 [-0.27; 0.04]	0.36
	Lipid Pathway PRS * Time	0.01 [-0.03; 0.05]	0.94	-0.01 [-0.03; 0.01]	0.69
	ARMS2	-0.22 [-0.50; 0.06]	0.34	-0.16 [-0.31; 0.00]	0.16
	ARMS2 * Time	-0.02 [-0.07; 0.02]	0.56	-0.02 [-0.04; 0.01]	0.39
RPE-BM	Complement Pathway PRS**	-0.10 [-0.35; 0.15]	0.69	-0.21 [-0.38; -0.05]	0.05
	Complement Pathway PRS * Time	0.05 [0.004; 0.09]	0.11	0.04 [0.02; 0.06]	3.23×10^{-5}
	Extracellular Matrix Pathway PRS***	0.002 [-0.24; 0.25]	0.99	-0.02 [-0.18; 0.14]	0.94
	Extracellular Matrix Pathway PRS * Time	-0.01 [-0.05; 0.03]	0.93	0.002 [-0.01; 0.02]	0.94
	Lipid Pathway PRS****	0.004 [-0.24; 0.25]	0.99	-0.09 [-0.25; 0.07]	0.56
	Lipid Pathway PRS * Time	-0.001 [-0.04; 0.04]	0.99	0.03 [0.02; 0.05]	3.74×10^{-4}
	ARMS2	0.18 [-0.06; 0.43]	0.34	0.15 [-0.02; 0.31]	0.23
	ARMS2 * Time	0.003 [-0.04; 0.05]	0.99	0.03 [0.02; 0.05]	0.002

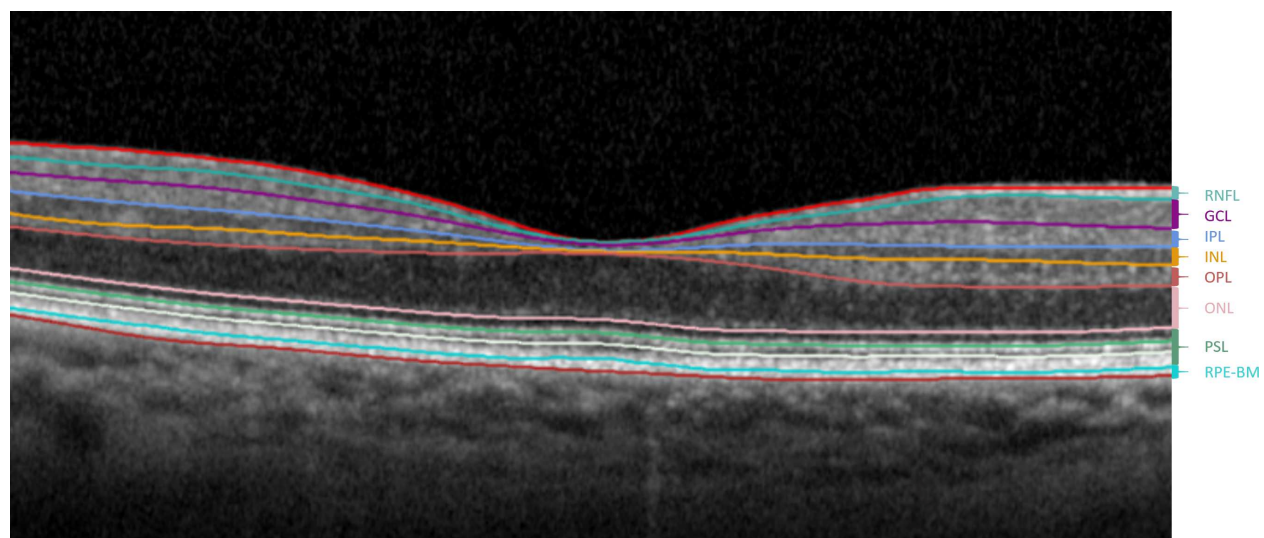
Mixed linear models are adjusted for age, sex and follow-up time. β in μm for 1-SD increase in PRS and in $\mu\text{m} / \text{year}$ for 1-SD increase in PRS for the interaction with follow-up time. ETDRS: Early Treatment Diabetes Retinopathy Study, CI: confidence interval, FDR: false discovery rate, SD: standard deviation, ONL: outer nuclear layer, PSL: photoreceptor segment layer, RPE-BM: retinal pigment epithelium- Bruch's Membrane complex, SD: standard deviation.

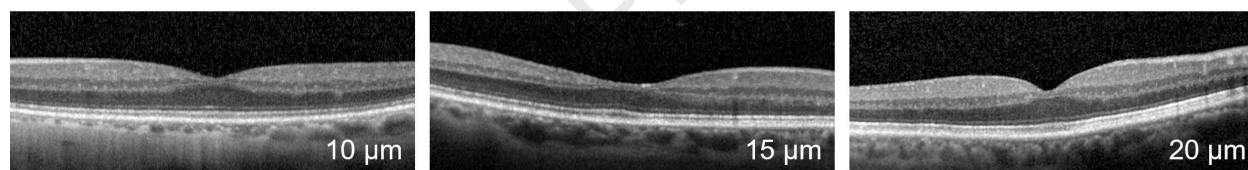
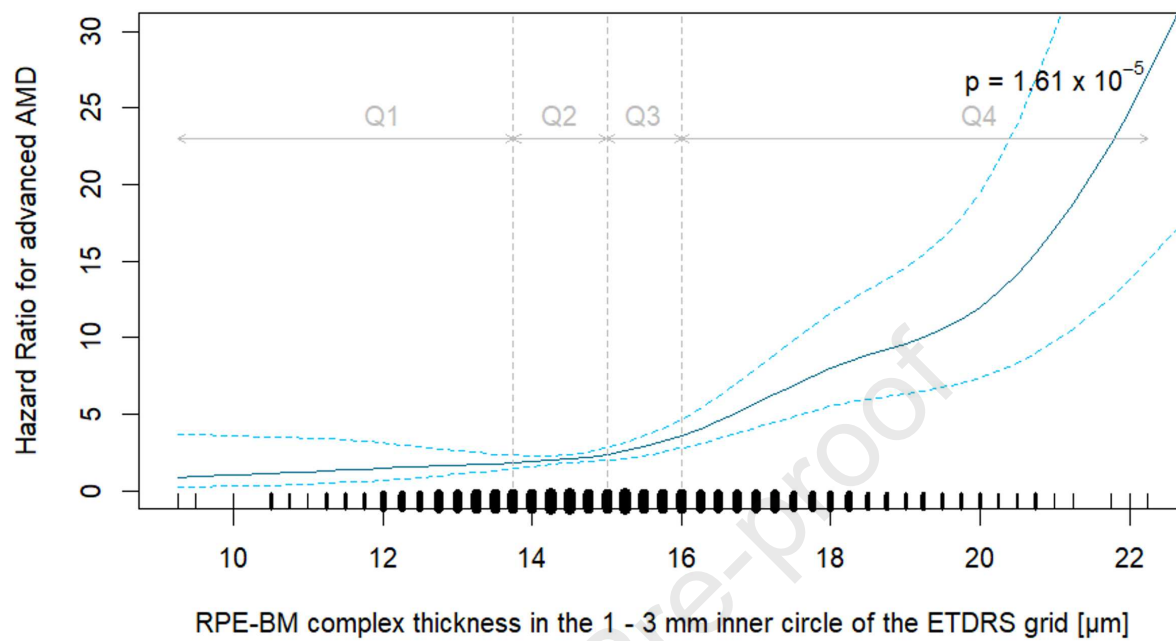
*For the inner circle, the thickness was calculated as the average of the nasal/temporal/superior/inferior subfields.

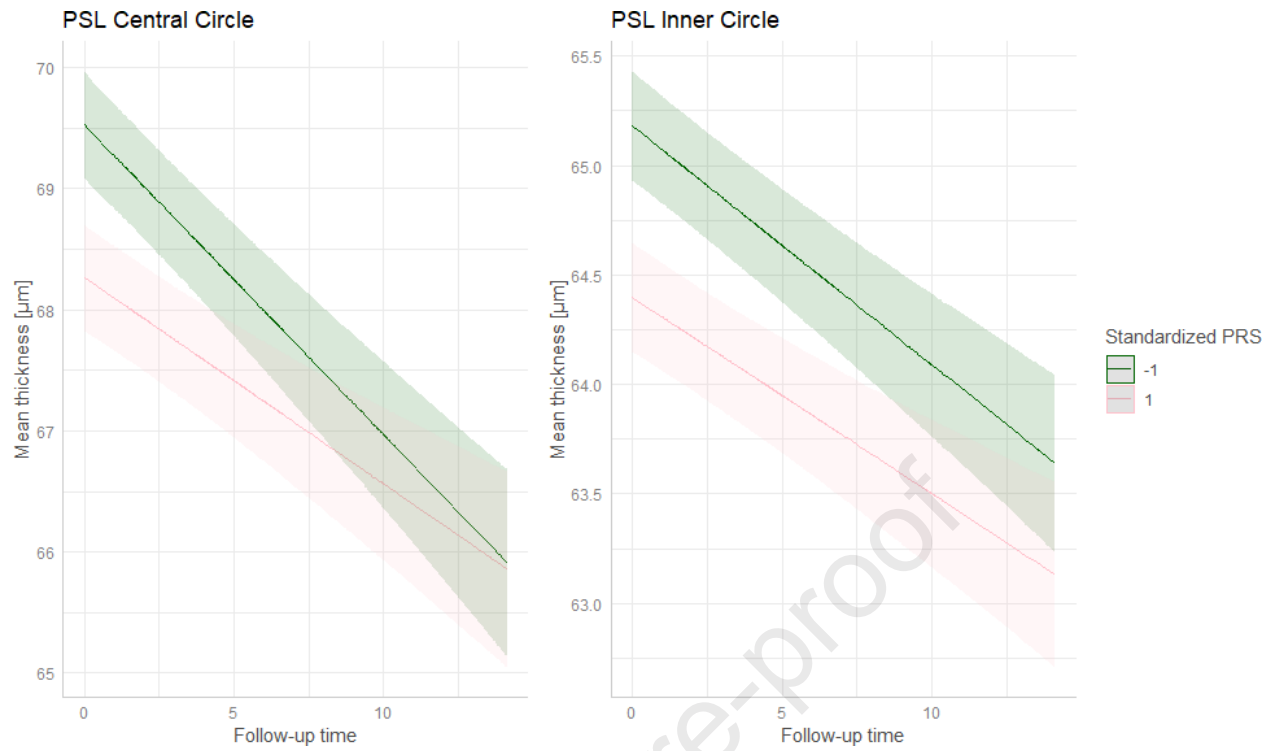
**The Complement Pathway is based on the following genes: C2, C3, C9, CFH, CFI, TMEM97.

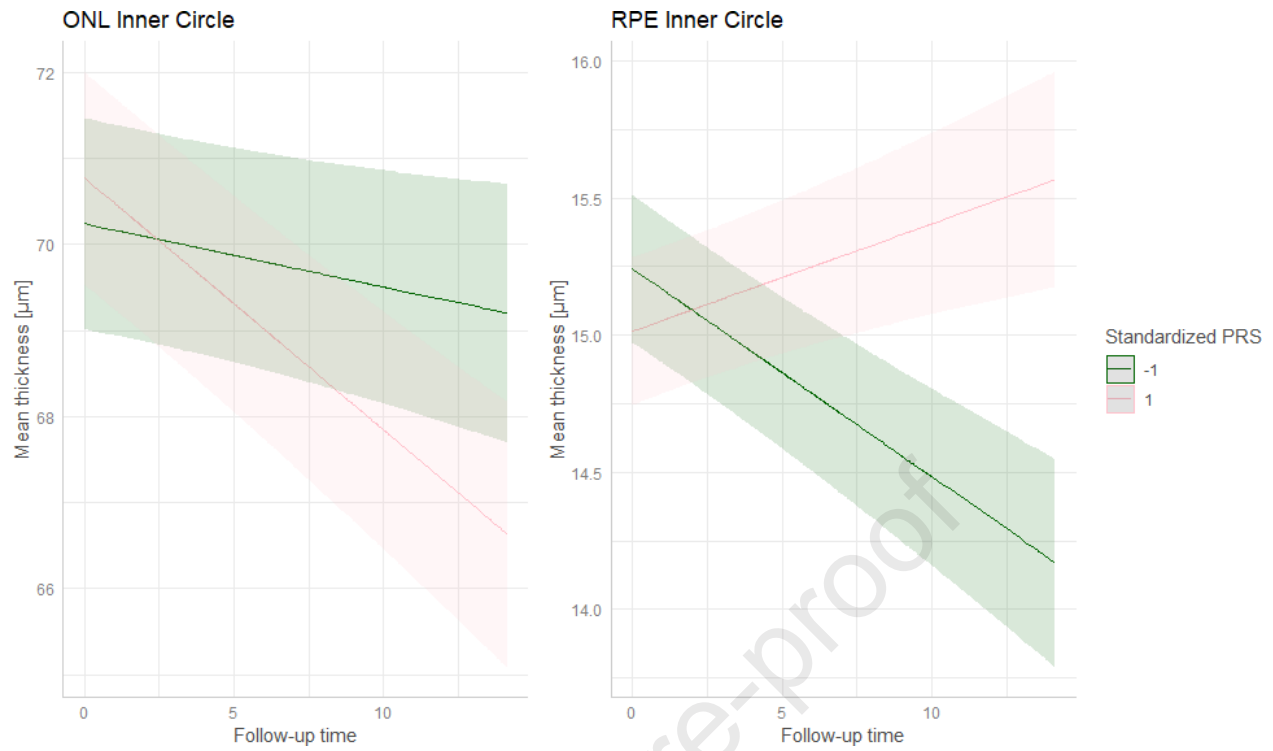
***The Extracellular Matrix Pathway is based on the following genes: ADAMTS9, COL4A3, COL8A1, SYN3, VEGFA.

****The Lipid Pathway is based on the following genes: ABCA1, APOE, CETP, LIPC.









Précis

A strong association of RPE-BM complex thickening with both incident AMD and the genetic risk for AMD was evidenced, highlighting its pivotal role in the clinical course of AMD.

Figure S2

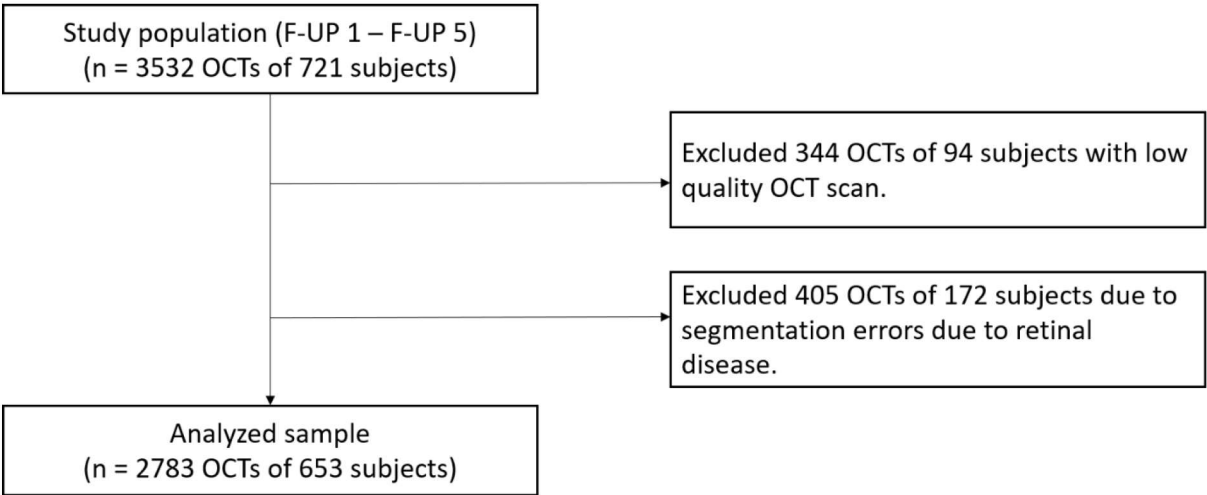


Figure S2. Flow chart showing selection of participants for analyses.
F-UP: Follow-Up Examination.

Figure S4

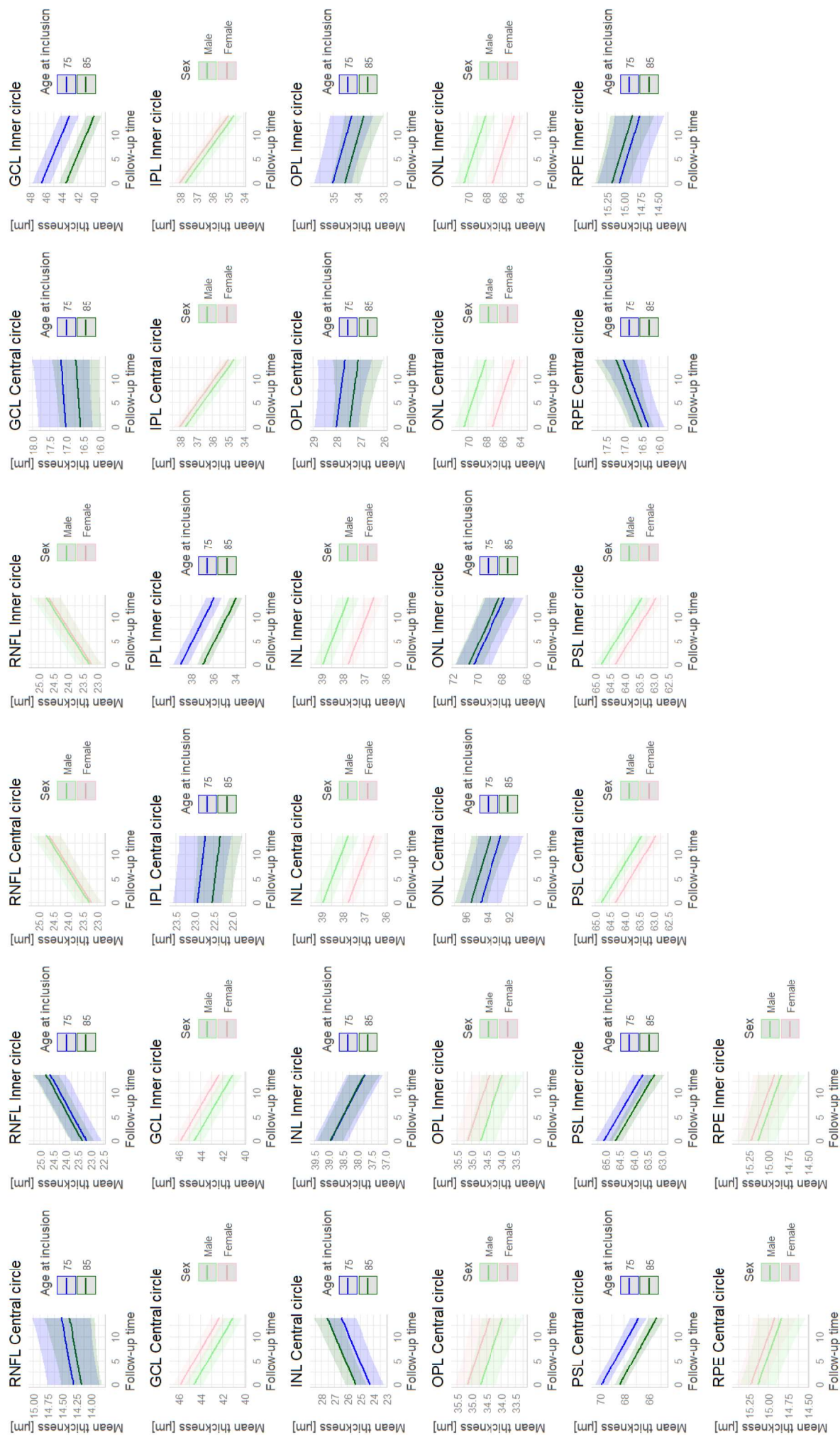


Figure S4. The evolution of macular layer thicknesses over the follow-up time for both the 1 mm central and the 1 – 3 mm inner circle of the Early Treatment Diabetic Retinopathy Study (ETDRS) grid as predicted by linear mixed models shown for the longitudinal relationship with sex and age, Alienor study, 2009–2024 (n = 2780 examinations of 653 participants). RNFL: retinal nerve fiber layer, GCL: ganglion cell layer, IPL: inner plexiform layer, INL: inner nuclear layer, OPL: outer plexiform layer, ONL: outer nuclear layer, PSL: photoreceptor segment layer, RPE-BM: retinal pigment epithelium- Bruch's Membrane complex.

Table S2. Macular layer thicknesses in Mean ± SD [μm] of the Study Population at baseline OCT examination (n = 1209 eyes of 653 participants).

Retinal Layer	Central Circle (1mm) of the ETDRS Grid	Inner Circle (1mm-3mm) of the ETDRS Grid*
RNFL	13.4 ± 3.0	23.1 ± 3.6
GCL	15.6 ± 5.0	45.1 ± 7.1
IPL	21.5 ± 4.1	37.6 ± 4.3
INL	22.6 ± 6.9	38.3 ± 3.8
OPL	27.5 ± 6.3	34.5 ± 4.8
ONL	94.3 ± 11.8	69.0 ± 9.0
PSL	68.5 ± 3.7	64.5 ± 2.0
RPE -BM	16.7 ± 2.9	15.1 ± 1.9
IRL	121.8 ± 12.2	247.5 ± 17.4
ORL	85.2 ± 4.7	79.6 ± 2.9
TRL	280.1 ± 24.9	327.1 ± 17.9

*For the inner circle, the thickness was calculated as the average nasal/temporal/superior/inferior subfields.
ETDRS: Early Treatment Diabetic Retinopathy Study, SD: standard deviation, RNFL: retinal nerve fiber layer, GCL: ganglion cell layer, IPL: inner plexiform layer, INL: inner nuclear layer, OPL: outer plexiform layer, ONL: outer nuclear layer, PSL: photoreceptor segment layer, RPE-BM: retinal pigment epithelium- Bruch's Membrane complex, IRL: inner retinal layers, ORL: outer retinal layers, TRL: total retinal layers.

Table S4. Longitudinal change of retinal layer thicknesses according to sex, age and time, Alienor study, 2009–2024. (n = 2780 examinations of 653 participants)

Retinal Layer		Central Circle of the ETDRS Grid		Inner Circle of the ETDRS Grid*	
		β [95% CI], μm	P_{FDR}	β [95% CI], μm	P_{FDR}
RNFL	Sex	-1.13 [-1.53; -0.73]	1.79×10^{-7}	-0.08 [-0.62; 0.46]	0.79
	Age at baseline (year)	-0.01 [-0.06; 0.03]	0.63	0.02 [-0.04; 0.08]	0.63
	Follow-up time (year)	0.01 [-0.01; 0.04]	0.30	0.10 [0.09; 0.12]	1.92×10^{-15}
GCL	Sex	-1.62 [-2.34; -0.90]	3.33×10^{-5}	1.25 [0.21; 2.28]	0.04
	Age at baseline (year)	-0.04 [-0.13; 0.04]	0.40	-0.31 [-0.43; -0.19]	1.47×10^{-6}
	Follow-up time (year)	0.01 [-0.02; 0.04]	0.53	-0.25 [0.27; -0.23]	1.92×10^{-15}
IPL	Sex	-1.75 [-2.33; -1.17]	3.12×10^{-8}	0.35 [-0.28; 0.99]	0.37
	Age at baseline (year)	-0.04 [-0.11; 0.03]	0.37	-0.20 [-0.27; -0.13]	1.79×10^{-7}
	Follow-up time (year)	-0.02 [-0.04; 0.01]	0.35	-0.22 [-0.23; -0.20]	1.92×10^{-15}
INL	Sex	-3.90 [-4.83; -2.97]	8.88×10^{-15}	-1.20 [-1.73; -0.68]	2.67×10^{-5}
	Age at baseline (year)	0.11 [0.00; 0.22]	0.09	0.002 [-0.06; 0.06]	0.95
	Follow-up time (year)	0.16 [0.11; 0.20]	7.20×10^{-10}	-0.09 [-0.11; -0.06]	3.00×10^{-11}
OPL	Sex	0.55 [-0.30; 1.39]	0.34	0.44 [-0.22; 1.12]	0.33
	Age at baseline (year)	-0.06 [-0.15; 0.04]	0.37	-0.05 [-0.12; 0.03]	0.35
	Follow-up time (year)	-0.03 [-0.09; 0.03]	0.51	-0.05 [-0.09; -0.01]	0.02
ONL	Sex	-2.48 [-4.19; -0.76]	0.01	-3.37 [-4.70; -2.03]	3.23×10^{-6}
	Age at baseline (year)	0.09 [-0.10; 0.29]	0.46	0.04 [-0.11; 0.19]	0.63
	Follow-up time (year)	-0.13 [0.21; -0.05]	2.58×10^{-3}	-0.18 [-0.23; -0.13]	3.91×10^{-11}
PSL	Sex	-0.09 [-0.57; 0.38]	0.73	-0.48 [-0.76; -0.21]	1.56×10^{-3}
	Age at baseline (year)	-0.15 [-0.20; -0.09]	1.32×10^{-6}	-0.04 [-0.07; -0.01]	0.02
	Follow-up time (year)	-0.21 [-0.25; -0.18]	1.92×10^{-15}	-0.10 [-0.12; -0.08]	1.92×10^{-15}
RPE-BM	Sex	0.23 [-0.17; 0.63]	0.37	0.09 [-0.20; 0.38]	0.62
	Age at baseline (year)	0.02 [-0.03; 0.07]	0.51	0.01 [-0.02; 0.04]	0.61
	Follow-up time (year)	0.05 [0.01; 0.09]	0.02	-0.02 [-0.04; -0.01]	0.02

*For the inner circle, the thickness was calculated as the average nasal/temporal/superior/inferior subfields.

ETDRS: Early Treatment Diabetic Retinopathy Study, CI: confidence interval, FDR: false discovery rate, RNFL: retinal nerve fiber layer, GCL: ganglion cell layer, IPL: inner plexiform layer, INL: inner nuclear layer, OPL: outer plexiform layer, ONL: outer nuclear layer, PSL: photoreceptor segment layer, RPE-BM: retinal pigment epithelium- Bruch's Membrane complex.

Table S5. Longitudinal change of macular layer thicknesses according to PRS, Alienor study, 2009–2024. (n = 2780 examinations of 653 participants)

Retinal Layer	Central Circle of the ETDRS Grid		Inner Circle of the ETDRS Grid*		P _{FDR}
	β [95% CI]	P _{FDR}	β [95% CI]	P _{FDR}	
RNFL	PRS	0.03 [-0.16; 0.22]	0.94	-0.15 [-0.41; 0.11]	0.53
	PRS* Follow-up time	0.002 [-0.02; 0.02]	0.96	-0.01 [-0.03; 0.01]	0.38
GCL	PRS	0.04 [-0.30; 0.39]	0.94	0.26 [-0.24; 0.75]	0.58
	PRS* Follow-up time	-0.004 [-0.03; 0.02]	0.94	-0.02 [-0.04; 0.00]	0.25
IPL	PRS	0.12 [-0.16; 0.40]	0.69	0.06 [-0.24; 0.36]	0.93
	PRS* Follow-up time	-0.01 [-0.03; 0.02]	0.88	-0.004 [-0.02; 0.01]	0.88
INL	PRS	0.003 [-0.45; 0.45]	0.99	-0.03 [-0.28; 0.22]	0.94
	PRS* Follow-up time	0.01 [-0.03; 0.06]	0.84	-0.01 [-0.03; 0.02]	0.72
OPL	PRS	-0.22 [-0.64; 0.19]	0.56	-0.36 [-0.68; -0.04]	0.11
	PRS* Follow-up time	-0.01 [-0.07; 0.05]	0.94	0.03 [-0.01; 0.07]	0.31
ONL	PRS	0.09 [-0.73; 0.91]	0.94	0.27 [-0.37; 0.91]	0.69
	PRS* Follow-up time	-0.03 [-0.12; 0.05]	0.69	-0.11 [-0.16; -0.06]	3.08 x 10⁻⁴
PSL	PRS	-0.63 [-0.86; -0.40]	2.42 x 10⁻⁶	-0.39 [-0.52; -0.26]	2.46 x 10⁻⁷
	PRS* Follow-up time	0.04 [0.00; 0.08]	0.11	0.01 [-0.01; 0.03]	0.58
RPE-BM	PRS	-0.005 [-0.21; 0.20]	0.99	-0.11 [-0.25; 0.03]	0.29
	PRS* Follow-up time	0.05 [0.01; 0.08]	0.08	0.06 [0.04; 0.07]	1.61 x 10⁻¹⁰

*For the inner circle, the thickness was calculated as the average the nasal/temporal/superior/inferior subfields. The fixed effects for all models were age, sex and follow-up time.

β in μm for 1-SD increase in PRS and in μm/year for 1-SD increase in PRS for the interaction with follow-up time.

ETDRS: Early Treatment Diabetic Retinopathy Study, CI: confidence interval, FDR: false discovery rate, PRS: polygenic risk score, RNFL: retinal nerve fiber layer, GCL: ganglion cell layer, IPL: inner plexiform layer, INL: inner nuclear layer, OPL: outer plexiform layer, ONL: outer nuclear layer, PSL: photoreceptor segment layer, RPE-BM: retinal pigment epithelium- Bruch’s Membrane complex, SD: standard deviation.

Table S6. Sensitivity analysis including AMD stage at baseline as fixed effect - Longitudinal change of retinal layer thicknesses according to PRS, Alienor study, 2009–2024. (n = 2780 examinations of 653 participants)

Retinal Layer	Central Circle of the ETDRS Grid		Inner Circle of the ETDRS Grid*		P _{FDR}
	β [95% CI]	P _{FDR}	β [95% CI]	P _{FDR}	
RNFL	PRS	0.04 [-0.16; 0.23]	0.96	-0.09 [-0.35; 0.18]	0.83
	PRS* Follow-up time	0.004 [-0.02; 0.03]	0.96	-0.01 [-0.03; 0.01]	0.52
GCL	PRS	0.04 [-0.32; 0.40]	0.99	0.20 [-0.31; 0.70]	0.83
	PRS* Follow-up time	-0.002 [-0.03; 0.03]	0.99	-0.02 [-0.05; 0.00]	0.26
IPL	PRS	0.08 [-0.21; 0.37]	0.83	0.005 [-0.30; 0.31]	0.99
	PRS* Follow-up time	-0.004 [-0.03; 0.02]	0.99	-0.01 [-0.02; 0.01]	0.83
INL	PRS	0.001 [-0.46; 0.47]	0.99	-0.02 [-0.28; 0.25]	0.99
	PRS* Follow-up time	0.01 [-0.06; 0.07]	0.83	-0.01 [-0.03; 0.02]	0.83
OPL	PRS	-0.16 [-0.59; 0.27]	0.83	-0.33 [-0.66; 0.00]	0.22
	PRS* Follow-up time	0.001 [-0.06; 0.06]	0.99	0.04 [0.00; 0.08]	0.26
ONL	PRS	0.08 [-0.78; 0.94]	0.99	0.34 [-0.32; 1.0]	0.76
	PRS* Follow-up time	-0.03 [-0.11; 0.05]	0.83	-0.12 [-0.17; -0.06]	1.28 x 10⁻⁴
PSL	PRS	-0.55 [-0.79; -0.32]	5.23 x 10⁻⁵	-0.39 [-0.53; -0.26]	2.78 x 10⁻⁷
	PRS* Follow-up time	0.04 [0.00; 0.08]	0.19	0.01 [-0.01; 0.03]	0.52
RPE-BM	PRS	-0.06 [-0.27; 0.14]	0.83	-0.13 [-0.27; 0.01]	0.26
	PRS* Follow-up time	0.05 [0.01; 0.09]	0.11	0.06 [0.04; 0.07]	7.42 x 10⁻¹¹

*For the inner circle, the thickness was calculated as the average the nasal/temporal/superior/inferior subfields. The fixed effects for all models were age, sex, follow-up time and AMD stage at baseline (no/early/intermediate/advanced).

β in μm for 1-SD increase in PRS and in μm/year for 1-SD increase in PRS for the interaction with follow-up time.

ETDRS: Early Treatment Diabetic Retinopathy Study, CI: confidence interval, FDR: false discovery rate, PRS: polygenic risk score, RNFL: retinal nerve fiber layer, GCL: ganglion cell layer, IPL: inner plexiform layer, INL: inner nuclear layer, OPL: outer plexiform layer, ONL: outer nuclear layer, PSL: photoreceptor segment layer, RPE-BM: retinal pigment epithelium- Bruch’s Membrane complex, SD: standard deviation.

Table S7. Sensitivity analysis after exclusion of all participants with any baseline AMD diagnosis - Longitudinal change of retinal layer thicknesses according to PRS, Alienor study, 2009–2024. (n = 1339 examinations of 405 participants)

Retinal Layer	Central Circle of the ETDRS Grid		Inner Circle of the ETDRS Grid*		P FDR
	β [95% CI]	P FDR	β [95% CI]	P FDR	
RNFL	PRS	0.03 [-0.24; 0.29]	0.91	-0.30 [-0.63; 0.04]	0.22
	PRS* Follow-up time	0.01 [-0.02; 0.04]	0.86	-0.02 [-0.05; 0.01]	0.34
GCL	PRS	-0.11 [-0.57; 0.34]	0.91	0.07 [-0.57; 0.72]	0.91
	PRS* Follow-up time	-0.03 [-0.07; 0.02]	0.42	-0.01 [-0.04; 0.02]	0.91
IPL	PRS	0.07 [-0.31; 0.44]	0.91	0.02 [-0.38; 0.41]	0.93
	PRS* Follow-up time	-0.02 [-0.06; 0.02]	0.56	-0.004 [-0.03; 0.02]	0.91
INL	PRS	-0.08 [-0.66; 0.50]	0.91	0.03 [-0.31; 0.37]	0.91
	PRS* Follow-up time	0.01 [-0.06; 0.07]	0.91	-0.03 [-0.06; 0.01]	0.28
OPL	PRS	-0.62 [-1.1; -0.09]	0.08	-0.42 [-0.84; 0.01]	0.17
	PRS* Follow-up time	0.05 [-0.03; 0.14]	0.43	0.01 [-0.05; 0.06]	0.91
ONL	PRS	0.86 [-0.13; 1.9]	0.22	1.0 [0.31; 1.8]	0.02
	PRS* Follow-up time	-0.06 [-0.18; 0.06]	0.56	-0.08 [-0.15; -0.02]	0.06
PSL	PRS	-0.61 [-0.93; -0.30]	1.22 x 10⁻³	-0.36 [-0.54; -0.18]	9.32 x 10⁻⁴
	PRS* Follow-up time	0.05 [-0.01; 0.11]	0.22	-0.003 [-0.03; 0.03]	0.91
RPE-BM	PRS	-0.37 [-0.58; -0.16]	3.12 x 10⁻³	-0.34 [-0.48; -0.20]	7.20 x 10⁻⁵
	PRS* Follow-up time	0.09 [0.04; 0.13]	2.31 x 10⁻³	0.05 [0.03; 0.07]	8.69 x 10⁻⁵

*For the inner circle, the thickness was calculated as the average the nasal/temporal/superior/inferior subfields. The fixed effects for all models were age, sex and follow-up time.

β in μm for 1-SD increase in PRS and in μm/year for 1-SD increase in PRS for the interaction with follow-up time.
ETDRS: Early Treatment Diabetic Retinopathy Study, CI: confidence interval, FDR: false discovery rate, PRS: polygenic risk score, RNFL: retinal nerve fiber layer, GCL: ganglion cell layer, IPL: inner plexiform layer, INL: inner nuclear layer, OPL: outer plexiform layer, ONL: outer nuclear layer, PSL: photoreceptor segment layer, RPE-BM: retinal pigment epithelium- Bruch’s Membrane complex, SD: standard deviation.

Table S8. Sensitivity analysis after exclusion of all participants with baseline intermediate or advanced AMD - Longitudinal change of retinal layer thicknesses according to PRS, Alienor study, 2009–2024. (n = 2174 examinations of 547 participants)

Retinal Layer	Central Circle of the ETDRS Grid		Inner Circle of the ETDRS Grid*		P FDR
	β [95% CI]		β [95% CI]		
RNFL	PRS	0.01 [-0.21; 0.23]	0.95	-0.16 [-0.44; 0.13]	0.48
	PRS* Follow-up time	0.002 [-0.02; 0.03]	0.83	-0.01 [-0.04; 0.01]	0.39
GCL	PRS	-0.07 [-0.46; 0.32]	0.81	0.30 [-0.25; 0.84]	0.48
	PRS* Follow-up time	-0.02 [-0.05; 0.01]	0.48	-0.02 [-0.05; 0.00]	0.26
IPL	PRS	0.12 [-0.20; 0.43]	0.62	0.17 [-0.16; 0.50]	0.48
	PRS* Follow-up time	-0.01 [-0.04; 0.02]	0.61	-0.01 [-0.03; 0.01]	0.38
INL	PRS	-0.14 [-0.64; 0.36]	0.72	0.03 [-0.25; 0.32]	0.89
	PRS* Follow-up time	0.01 [-0.04; 0.07]	0.72	-0.02 [-0.05; 0.01]	0.38
OPL	PRS	-0.18 [-0.63; 0.28]	0.61	-0.39 [-0.75; -0.04]	0.11
	PRS* Follow-up time	-0.005 [-0.07; 0.06]	0.93	0.02 [-0.03; 0.06]	0.64
ONL	PRS	0.47 [-0.41; 1.4]	0.48	0.57 [-0.11; 1.3]	0.30
	PRS* Follow-up time	-0.06 [-0.16; 0.03]	0.38	-0.10 [-0.16; -0.04]	0.004
PSL	PRS	-0.57 [-0.84; -0.31]	2.10 x 10⁻⁴	-0.40 [-0.55; -0.26]	4.00 x 10⁻⁵
	PRS* Follow-up time	0.05 [0.01; 0.10]	0.06	0.01 [-0.01; 0.03]	0.48
RPE-BM	PRS	-0.28 [-0.45; -0.12]	0.004	-0.31 [-0.43; -0.19]	1.28 x 10⁻⁵
	PRS* Follow-up time	0.05 [0.02; 0.09]	9.14 x 10⁻³	0.04 [0.02; 0.05]	2.89 x 10⁻⁵

* For the inner circle, the thickness was calculated as the average the nasal/temporal/superior/inferior subfields. The fixed effects for all models were age, sex and follow-up time.

β in μm for 1-SD increase in PRS and in μm/year for 1-SD increase in PRS for the interaction with follow-up time.

ETDRS: Early Treatment Diabetic Retinopathy Study, CI: confidence interval, FDR: false discovery rate, PRS: polygenic risk score, RNFL: retinal nerve fiber layer, GCL: ganglion cell layer, IPL: inner plexiform layer, INL: inner nuclear layer, OPL: outer plexiform layer, ONL: outer nuclear layer, PSL: photoreceptor segment layer, RPE-BM: retinal pigment epithelium- Bruch’s Membrane complex, SD: standard deviation.

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3.3.3 Esterified 3-OH FAs exposure and retinal layer thicknesses

Using linear mixed models, we analyzed the longitudinal association of each macular layer thickness with longstanding endotoxin-exposure as measured by esterified 3-OH FAs (**Table 2, Figure 16 and 17**). We observed an association between esterified 3-OH FAs and thinner baseline IRL and TRL in the inner circle of the ETDRS grid ($\beta = -1.82 \mu\text{m}$ for 1-SD increase, 95 % CI [-3.10; -0.55] and $\beta = -1.98 \mu\text{m}$, 95 % CI [-3.29; -0.67], respectively). However, none of the results was significant after applying the Benjamini-Hochberg procedure to control for the false discovery rate ($P_{\text{FDR}} = 0.12$ for both).

*Table 2. Longitudinal change of retinal layer thicknesses according to 3-OH FAs
Alienor study, 2006–2024. (n = 2780 examinations of 653 participants)*

Retinal Layer	Central Circle of the ETDRS Grid		Inner Circle of the ETDRS Grid*	
	β [95 % CI]	P_{FDR}	β [95 % CI]	P_{FDR}
RNFL				
3-OH FAs	-0.17 [-0.37; 0.03]	0.31	-0.16 [-0.43; 0.11]	0.43
3-OH FAs* Follow-up time	-0.01 [-0.04; 0.01]	0.31	-0.01 [-0.01; 0.03]	0.49
GCL				
3-OH FAs	-0.15 [-0.51; 0.20]	0.49	-0.47 [-0.98; 0.04]	0.31
3-OH FAs* Follow-up time	0.02 [-0.01; 0.04]	0.31	-0.003 [-0.03; 0.02]	0.84
IPL				
3-OH FAs	-0.21 [-0.50; 0.08]	0.31	-0.38 [-0.69; -0.07]	0.19
3-OH FAs* Follow-up time	0.01 [-0.01; 0.04]	0.44	-0.01 [-0.03; 0.01]	0.49
INL				
3-OH FAs	-0.14 [-0.61; 0.32]	0.63	-0.15 [-0.41; 0.11]	0.43
3-OH FAs* Follow-up time	0.002 [-0.05; 0.04]	0.92	-0.03 [-0.05; -0.01]	0.17
OPL				
3-OH FAs	-0.05 [-0.47; 0.38]	0.85	-0.19 [-0.52; 0.14]	0.43
3-OH FAs* Follow-up time	0.01 [-0.05; 0.07]	0.82	0.02 [-0.02; 0.05]	0.49
ONL				
3-OH FAs	-0.77 [-1.62; 0.08]	0.31	-0.49 [-1.15; 0.18]	0.31
3-OH FAs* Follow-up time	-0.06 [-0.14; 0.01]	0.31	-0.04 [-0.09; 0.01]	0.31
PSL				
3-OH FAs	-0.19 [-0.43; 0.05]	0.31	-0.95 [-0.23; 0.04]	0.34
3-OH FAs* Follow-up time	0.02 [-0.02; 0.05]	0.49	0.78 [-0.01; 0.03]	0.49
RPE				
3-OH FAs	-0.02 [-0.23; 0.18]	0.85	-0.62 [-0.21; 0.08]	0.49
3-OH FAs* Follow-up time	0.04 [0.00; 0.07]	0.30	0.02 [0.00; 0.03]	0.21
IRL				
3-OH FAs	-1.46 [-3.22; 0.30]	0.31	-1.82 [-3.10; -0.55]	0.12
3-OH FAs* Follow-up time	-0.04 [-0.13; 0.05]	0.49	-0.06 [-0.13; 0.01]	0.31
ORL				
3-OH FAs	-0.21 [-0.53; 0.11]	0.36	-0.16 [-0.38; 0.05]	0.31

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	3-OH FAs* Follow-up time	0.05 [0.00; 0.10]	0.21	0.02 [0.00; 0.05]	0.21
TRL					
	3-OH FAs	-1.67 [-3.43; 0.10]	0.31	-1.98 [-3.29; -0.67]	0.12
	3-OH FAs* Follow-up time	0.01 [-0.08; 0.11]	0.85	-0.03 [-0.10; 0.04]	0.49

*For the inner circle, the thickness was calculated as the average the nasal/temporal/superior/inferior subfields.

The fixed effects for all models were age, sex and follow-up time.

β in μm for 1-SD increase in 3-OH FAs and in $\mu\text{m}/\text{year}$ for 1-SD increase in 3-OH FAs for the interaction with follow-up time. 3-OH FAs were standardized using the formula $Z = (x - \mu) / \sigma$, where Z is the standard score, μ the mean and σ the standard deviation of 3-OH FAs, respectively.

ETDRS: Early Treatment Diabetic Retinopathy Study, CI: confidence interval, FDR: false discovery rate, 3-OH FAs: 3-hydroxy fatty acids, RNFL: retinal nerve fiber layer, GCL: ganglion cell layer, IPL: inner plexiform layer, INL: inner nuclear layer, OPL: outer plexiform layer, ONL: outer nuclear layer, PSL: photoreceptor segments layer, RPE-BM: retinal pigment epithelium- Bruch's Membrane complex, IRL: inner retinal layers, ORL: outer retinal layers, TRL: total retinal layers, SD: standard deviation.

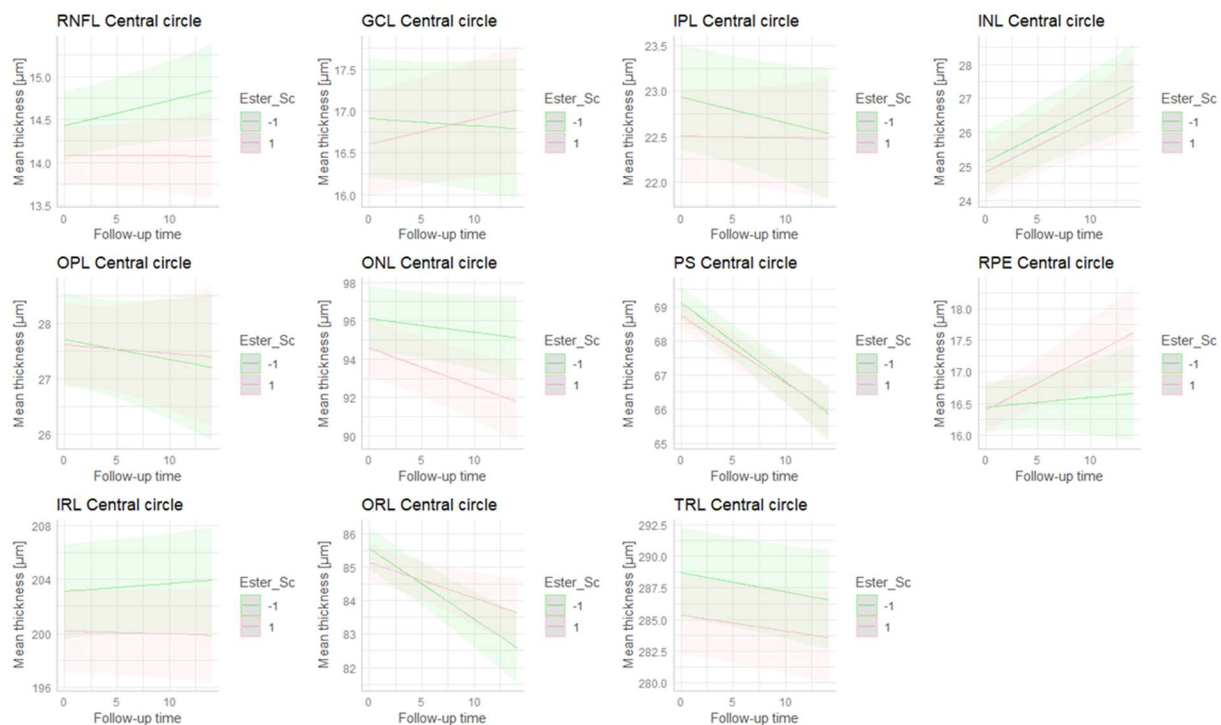


Figure 16. Evolution of macular layer thicknesses in the central circle according to LPS

Evolution of macular layer thicknesses over the follow-up time for the 1 mm central circle of the Early Treatment Diabetic Retinopathy Study (ETDRS) grid as predicted by linear mixed models shown for the longitudinal relationship with 3-OH FAs, Alienor study, 2009–2024 ($n = 2780$ examinations of 653 participants).

LPS: lipopolysaccharide, RNFL: retinal nerve fiber layer, GCL: ganglion cell layer, IPL: inner plexiform layer, INL: inner nuclear layer, OPL: outer plexiform layer, ONL: outer nuclear layer, PSL: photoreceptor segments layer, RPE-BM: retinal pigment epithelium- Bruch's Membrane complex, IRL: Inner retinal layers, ORL: Outer retinal layers, TRL: Total retinal layers.

Results

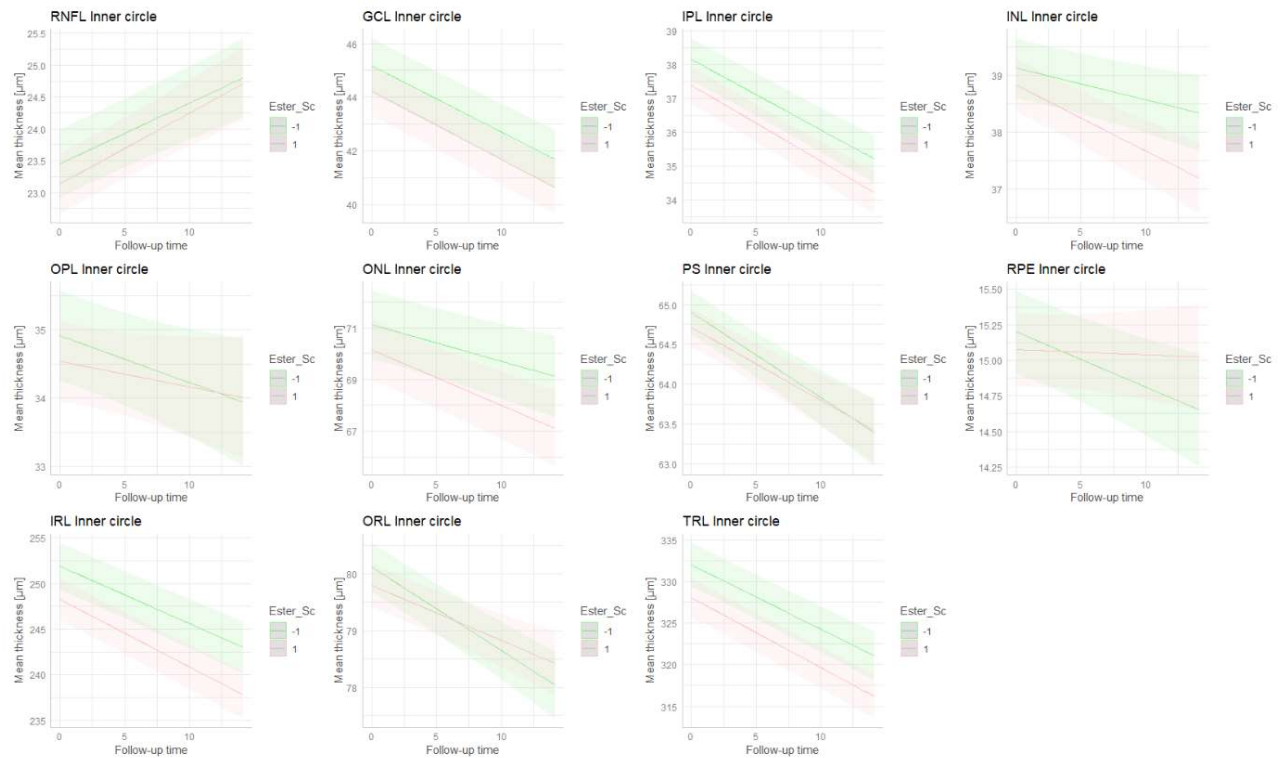


Figure 17. Evolution of macular layer thicknesses in the inner circle according to LPS

Evolution of macular layer thicknesses over the follow-up time for the 1–3 mm inner circle of the Early Treatment Diabetic Retinopathy Study (ETDRS) grid as predicted by linear mixed models shown for the longitudinal relationship with 3-OH FAs, Alienor study, 2009–2024 ($n = 2780$ examinations of 653 participants).

LPS: lipopolysaccharide, RNFL: retinal nerve fiber layer, GCL: ganglion cell layer, IPL: inner plexiform layer, INL: inner nuclear layer, OPL: outer plexiform layer, ONL: outer nuclear layer, PS: photoreceptor segments layer, RPE-BM: retinal pigment epithelium-Bruch's Membrane complex, IRL: Inner retinal layers, ORL: Outer retinal layers, TRL: Total retinal layers.

3.3.4 Discussion

The results of Article 3 demonstrate a strong link between foveal RPE-BM complex thickening and both incident intermediate and advanced AMD. Longitudinal thickening of the RPE-BM complex was strongly associated with the total AMD PRS, and in particular with the complement, lipid, and ARMS2 pathway PRS. Additionally, both the total PRS and complement-specific PRS were linked to thinner PSL at baseline and longitudinal ONL thinning.

The RPE-BM complex thickening observed in SD-OCT scans may reflect different changes within the RPE and BM including drusen, BLamDs, lipofuscin, or diffuse Bruch's membrane thickening. Our data shown in Article 3 underscore that thickening of the RPE-BM complex is a key imaging biomarker of AMD progression and align with the current literature.^{210,243} Our findings extend the reported cross-sectional RPE-BM thickening in AMD by showing that

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RPE-BM thickening is associated with future incidence of intermediate and advanced AMD, independent of potential confounders like baseline AMD stage.

A recent study highlighted the potential pathophysiologic sequence in morphological changes in the development of AMD, where PSL thinning starts more than a decade before RPE-BM complex thickening.²⁴² We observed that the total PRS for AMD was associated with longitudinal RPE-BM thickening and baseline PSL thinning, but not with longitudinal PSL thinning, which is in line with the proposed sequence of morphological changes in AMD.²⁴² Additionally, the total PRS was linked to ONL thinning, aligning with previous reports of ONL reduction in severe early AMD.^{210,243}

Our analysis of repeated SD-OCT measurements in individuals over 75 years of age allowed us to report longitudinal changes in macular layers by age and sex. We identified thinner inner macular layers in women, consistent with earlier cross-sectional studies.^{209,244}

Using specific genetic pathways influencing macular layer changes, we could show that the complement pathway was strongly associated with thinner baseline photoreceptor layer thinning as well as with longitudinal ONL thinning and RPE-BM thickening. For RPE-BM thickening, our analysis showed that a part from the complement pathway also the lipid pathway along with ARMS2 were implicated, indicating a multifaceted genetic influence on RPE-BM thickening in AMD.

The results on esterified 3 OH-FAs and thinner baseline IRL and TRL, even though not significant after accounting for the false discovery rate, seem intriguing nonetheless. They imply that low-grade inflammation may lead to certain morphological changes in the retina, potentially measurable in SD-OCT.

In conclusion, we report a strong association between RPE-BM thickening and incident intermediate and advanced AMD. Further, an association between the complement-specific polygenic risk score and thinner baseline PSL was evidenced indicating a complement driven mechanism in the early changes in AMD. Future research is needed to validate these findings and further unravel the mechanisms driving AMD, with the ultimate goal of developing more effective treatments for early stage AMD.

4 Discussion

4.1 Main results

In a population-based setting of a cohort of elderly adults, we could demonstrate several associations between 3-OH FAs and age-related eye-diseases:

- 3-OH FAs were significantly associated with incident early AMD (Article 1)
- 3-OH FAs were significantly associated with baseline RNFL thinning (Article 2)
- 3-OH FAs tended to be linked to macular changes quantifiable with SD-OCT

We also reported a strong link between thicker foveal RPE-BM complex and both incident intermediate and advanced AMD. Further, we could show a strong link between genetic susceptibility to AMD and morphologic changes in AMD (in particular RPE-BM complex thickening and PSL thinning, Article 3).

4.2 LPS and the aging retina

Our findings on a proxy of LPS contribute to the body of knowledge on inflammation and age-related eye diseases and align with current literature.

LPS is known for its immunostimulatory effects including an altered production of key inflammatory mediators, such as cytokines and chemokines. As LPS is naturally found in the human microbiome, microbial dysbiosis and other factors discussed in detail in section 1.3.3. lead to LPS translocation into the bloodstream. The choroid, which provides blood supply to the outer retina is one of the most vascularized tissues in the body. This rich vascularization is essential for supplying nutrients and oxygen to retinal cells, but also renders the retina a more likely target for circulating bacterial endotoxins like LPS, especially in case of vascular dysfunction or increased permeability in retinal vessels. Moreover, the high metabolic demand of retinal tissues makes them particularly vulnerable to inflammatory and oxidative damage triggered by LPS. Exposure to light, including photo-oxidative stress can further impair mitochondrial function, which is crucial for the survival of retinal neurons.

However, due to the origin of LPS as a bacterial component, it is hardly possible to distinguish between the direct effects of LPS and of other pathogenic factors of gram-negative bacteria, which implies also the possibility that elevated LPS might be a proxy for

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another bacterial pathogen or inflammation of bacterial origin. In fact, in experimental settings, LPS is often used to induce inflammatory responses of bacterial origin.^{232,234}

Still, LPS is understood to be a potent inflammatory stimulus itself and therefore circulating LPS has been hypothesized to play a role in AMD and glaucoma pathogenesis.

Thus, in the following, we will discuss the direct effects of LPS and its role according to its different sources in the light of our findings and the body of literature.

4.2.1 Direct effects of LPS in age-related eye diseases

LPS triggers a strong immune response, primarily by activating innate immune cells, which recognize it through pattern recognition receptors such as TLR4. In the following paragraphs, an overview on the known effects of LPS in AMD and POAG/retinal neurodegeneration are given.

4.2.1.1 LPS in AMD

LPS could provoke pro-inflammatory immune responses and RPE dysfunction leading to accumulation and deposition of undegradable byproducts of the visual cycle and eventually AMD. There are several pieces of evidence in the literature underscoring this hypothesis. An *in vitro* study demonstrated that LPS induces RPE-cytotoxicity via proinflammatory autocrine signalling.²²⁷ In a case-control setting, an altered B- cell antibody production in response to LPS in AMD patients compared to controls was reported, which potentially contributed to AMD pathogenesis.²²⁸ Further, LPS stimulation has been reported to increase messenger ribonucleic acid (mRNA) expression of IL-6, VEGF and TNF α via the TLR4 pathway.²³³ In addition, several studies report LPS induced increase of CNV lesions, via activation of the TLR4 pathway,²²⁹ accompanied by upregulation of VEGF,²³⁰ depending on retina-resident myeloid cells and independent of adaptive immunity.²³⁴ However, pre-treatment with LPS was also shown to decrease the size of experimental CNV by laser photocoagulation in mice via IL-10.²³¹

Although the above cited studies showed partly different results and impact on CNVs, all the discussed studies underline the direct immunomodulatory influence of LPS on changes

associated with AMD. The reported differences might be due to different LPS treatment regimen which may activate different signaling pathways determining the responsiveness and polarization of microglia/macrophages and thus explain the variability of experimental outcome.

However, contrary to the literature, we did not find an association of LPS with advanced AMD (Article 1), which was not separately analyzed for geographic atrophy and CNV due to low numbers of cases. This could be due to the following reasons: i) Hit and run theory: LPS triggers inflammation during the early pathogenic processes of AMD. The impact of LPS also unfolds its effect on the progression to advanced AMD, but is no longer detectable at the time of development of advanced AMD. ii) The time lag between blood draw in our study until the development of advanced AMD is too long. Therefore, no association between a plasma inflammation parameter measured in 1999 and advanced AMD, developing later and on the grounds of early AMD, can be found. iii) Since the number of participants with incident advanced AMD is lower than for early AMD, our analysis may have been underpowered to find an association.

4.2.1.2 LPS in retinal neurodegeneration / POAG

In retinal neurodegeneration, LPS is primarily thought to induce inflammation and neurotoxicity via microglia. *In vitro*, low-dose LPS led to enhancement of axonal degeneration and neuronal loss and was accompanied by microglial activation in the optic nerve and retina as well as upregulation of TLR4 signaling and the complement system.¹⁹⁰

Evidence on the association between endotoxin exposure and neurodegeneration arises in particular through studies on brain neurodegeneration, which however resembles retinal neurodegeneration in many respects. In the brain, different mechanisms for the contribution of LPS in neurodegeneration have been described. LPS is known to activate TLR4 on microglia to produce nitric oxide and pro-inflammatory cytokines.²⁴⁵ Combining LPS with interferon γ (IFN γ) (from recruited T cells), leads to release of nitric oxide that can kill neurons when combined with either hypoxia or superoxide from the NADPH oxidase.^{246,247} In neurodegenerative disease, neuronal loss is often preceded by synaptic loss.²⁴⁸ It has been shown that activation of the complement cascade by neuroinflammation drive synaptic loss

by microglial phagocytosis.²⁴⁹ In complement C3 deficient mice, the LPS induced activation of the classical complement system in the brain could be prevented.²⁵⁰ Clinical evidence showed that serum LPS was elevated in patients with Alzheimer's disease compared to cognitively healthy individuals.²⁵¹

In the context of retinal neurodegeneration, we report an inverse association of a proxy of LPS with baseline retinal nerve fiber thickness, but not with longitudinal change of retinal nerve fiber thickness (Article 2). Extrapolating these results to the potential direct effects of LPS, could imply that the temporal direct effects of LPS are limited to a defined time frame in relation to the blood draw, which took place about eight years before the first RNFL measurement in the Alienor study.

4.2.2 LPS as a marker for Gram-negative bacterial infection

Due to its origin as bacterial endotoxin, it is hardly possible to distinguish between the effects of LPS and of other pathogenic factors of gram-negative bacteria. Therefore, elevated LPS could well indicate the presence or involvement of Gram-negative bacteria themselves.

4.2.2.1 Gram-negative bacterial infection linked to AMD

Concerning Gram-negative bacterial infection and AMD, especially *Chlamydia pneumoniae* (*C. pneumoniae*) has been studied extensively in the literature. *C. pneumoniae* has a unique biphasic life cycle characterized by an obligate intracellular replicative and an extracellular infectious form. It may cause chronic infection in the respiratory tract, induce proinflammatory cytokines, dysregulate host-cell lipid metabolism, and modulate apoptotic pathways.²⁵² Such chronic systemic inflammation has been proposed to have an impact at sites distant from the infection and could therefore contribute to diseases with an inflammatory component such as AMD.

In the last decade of the 20th century, chronic inflammatory events and in particular infections by *C. pneumoniae* have been discussed in the pathogenesis of atherosclerosis after detection of *C. pneumoniae* in the plaques of the coronary and carotid arteries.²⁵³ The association between atherosclerosis and AMD made worth further investigation in the association of *C. pneumoniae* and AMD.^{254,255}

Discussion

A link between *C. pneumoniae* and AMD was first described in 2003, reporting increased antibody levels to *C. pneumoniae* in patients with AMD.^{256,257} However, in the following years, several other studies were published showing conflicting results.^{258–261} The reasons for the discrepancies remain unclear, but might be partly explainable by varied methodology, in particular lack of control groups, use of different analytical assays and inclusion criteria.

Concerning the underlying mechanisms, several hypotheses have been proposed. As the most common trigger of the alternative pathway are microbial in nature, *Chlamydia pneumoniae* could activate the alternative complement pathway. This may induce uncontrolled activation of the complement pathway, especially in individuals carrying the Y402H variant of the CFH gene, which leads to chronic inflammation and damage to the RPE-BM complex.^{262,263} Another hypothesis involves the intracellular infection of different cell types, including endothelial cells, macrophages, RPE cells and fibroblasts, by *C. pneumoniae*.²⁶⁴ Macrophages infected with *C. pneumoniae* may enter the bloodstream and establish a chronic infection ocular tissues and especially the choroid (given the particular attraction of *C. pneumoniae* for vascular tissue). Secretion of inflammatory cytokines may impair vascular architecture, which may lead to the development of AMD.²⁵⁶ This hypothesis is supported by the VEGF-secretion of macrophages after infection with *C. pneumoniae* and that the size of CNV is positively correlated with underlying *C. pneumoniae* infection.^{233,234,257,265}

However, also the “innocent bystander hypothesis”, where inactive *C. pneumoniae* arrives at already damaged macular tissues and are not a trigger of inflammation, needs to be considered as over 50 % of the adult population carry anti-*C. pneumoniae* antibodies, but only a fraction infected with *C. pneumoniae* progress to AMD.^{234,264,266}

Other bacteria have been studied more anecdotally in the context of AMD, but no significant results were found for *Chlamydia trachomatis*, *Escherichia coli*, *Mycoplasma pneumoniae*, *Chlamydia psittaci* and *Helicobacter pylori*.^{267–270}

In the context of *C. pneumoniae* and AMD, we cannot differentiate whether the measured LPS is related to *C. pneumoniae*. However, considering the body of literature on *C. pneumoniae* and AMD, it would be interesting to study LPS in AMD including serology on *C. pneumoniae*, which is currently not available in the Alienor study.

Finally, I would like to draw the attention of the interested reader to our review article entitled “Could infectious agents play a role in the onset of age-related macular degeneration?”, which is listed in the publication list of this thesis and can be found as Appendix 2.

4.2.2.2 Gram-negative bacterial infection linked to retinal neurodegeneration / POAG

Concerning Gram-negative bacterial infection and POAG, also one Gram-negative bacteria has been studied extensively: *Helicobacter pylori* (*H. pylori*).

H. pylori is a curved spiral gram-negative bacterium that colonizes the gastric mucosa and was described initially in 1983.²⁷¹ Globally, more than 40 % of the population are asymptomatically infected with *H. pylori*.²⁷² Persistent colonization with virulent strains are known to cause a number of gastric and extragastric disorders.²⁷³

H. pylori was primarily studied in the context of glaucoma because i) glaucoma and *H. pylori* are both linked with older age in the developed world, ii) *H. pylori* infection may induce the release of vasoactive and proinflammatory substances affecting the vascular tone and iii) *H. pylori* infection is associated with arteriosclerosis-induced increased platelet activation and aggregation.²⁷⁴ The first results on *H. pylori* and glaucoma were published in 2001 and were based on a cohort of POAG and pseudoexfoliation glaucoma patients and found an association for *H. pylori* seropositivity and glaucoma.²⁷⁴ The same authors reported that glaucoma patients with successful eradication of *H. pylori* benefitted in terms of regulation of intraocular pressure and visual field parameters.²⁷⁵ However, in 2003, a larger prospective case-control study found no conclusive association between *H. pylori* and POAG.²⁷⁶ Over the last two decades, several more studies were published exploring the link between *H. pylori* and glaucoma including at least three meta-analyses. The meta-analysis by Zeng et al., including 10 studies yielded a pooled odds ratio (OR) for *H. pylori* infection and OAG susceptibility of 2.08 (95 % CI, 1.42–3.04).²⁷⁷ The meta-analysis by Doulberis et al., including a total of 15 studies, found an overall association between *H. pylori* infection and glaucoma of pooled OR = 2.08 (CI 95 % 1.48–2.93).²⁷⁸ The meta-analysis by Amini et al. calculated separate OR for the 7 cohort studies (pooled OR = 1.59, 95 % CI; 1.36–1.87) and 17 case-control studies (pooled OR = 2.87, 95 % CI; 2.33–3.52).²⁷⁹ The results of these three meta

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analyses, although different in methodology and quality, underscore the positive association between active *H. pylori* infection and glaucoma.

From a pathogenetic point of view, potential links between *H. pylori* infection and glaucoma may include the release of pro-inflammatory and vasoactive substances including nitric oxide and formation of reactive oxygen species, which have been implicated neurotoxicity, optic nerve degeneration and apoptosis of retinal ganglionic cells.^{274,278,280} Nitric oxide due to its vasoactive activity could possibly imbalance the ocular vascular tone *leading to increased intraocular pressure*.²⁷⁴ *H. pylori* infection has also been reported to stimulate platelet and platelet-leukocyte aggregation and platelet activation and aggregation have been proposed to lead to decreased of ocular blood.^{274,280}

Other bacteria have been studied in the context of the human microbiome and/or in relation to secondary glaucoma.²⁸¹

In the context of *H. pylori* and glaucoma, we cannot differentiate whether the measured LPS is related to *H. pylori*. However, considering the body of literature on *H. pylori* and glaucoma, it would be interesting to study LPS and retinal neurodegeneration including serology on *H. pylori*, which is currently not available in the Alienor study.

4.2.3 LPS as a marker human microbiome dysbiosis

In the last years, the topic of the microbiome has gained attention in all areas of medicine. In the field of the microbiome and the eye, several dozen scientific articles have been published, including a considerable amount of reviews and hypothesis papers. When comparing published microbiome taxonomic data across different studies, one has to bear in mind that the field of metagenomics is still in its infancy.²⁸² Due to an extremely high level of transformation generational leaps in the technical capacities and analytical and computational capabilities may take place within a very short period, rendering it difficult to compare results of different studies.

In general, for the analysis of the microbiome, different analytical procedures are performed on a certain taxonomic level (**Figure 18**) to assess the α -diversity (describing the richness and evenness of different taxa within a sample) and β -diversity (comparing the differences in taxa across different hosts or samples).²⁸³

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Traditionally, the microbiome is compared at one certain taxonomy level, most commonly on the level of the genus, although modern computation also allows for comparison on species level.²⁸² Each level offers unique insights into the composition, diversity, and functional capacity of microbiomes across different conditions or hosts.²⁸⁴

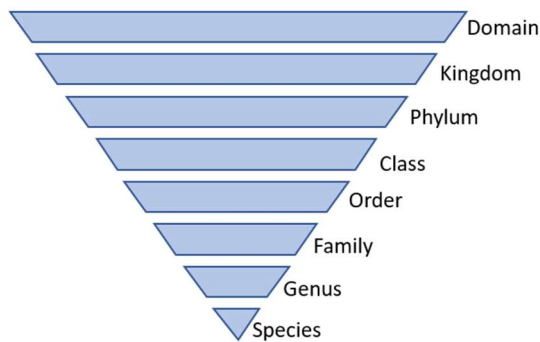


Figure 18. The eight major taxonomy levels in biology. Microbiomes are usually classified and compared based on taxonomic ranks.

4.2.3.1 The human microbiome and AMD

The gut microbiome

Regarding AMD and the human gut microbiota, only a handful original studies have been published, using DNA from faecal samples to analyze the gut microbial profile.^{285–289}

Although faecal samples are a relatively easily accessible source, it should be noted that the information obtained from faecal samples does not represent the complete picture within the gut.²⁹⁰ Still, the available studies have revealed differences in abundance of specific bacteria in patients with advanced AMD compared to healthy age-matched controls.

A study from Switzerland observed an enrichment in the genera *Anaerotruncus*, *Oscillibacter*, *Ruminococcus torques* and *Eubacterium ventriosum*; and an increased *Firmicutes/Bacteroidetes* ratio in the fecal microbiota of patients with nAMD (n = 12) compared with healthy controls (n = 11).²⁸⁵ Comparing the metabolic features of the microbiome, the pathways for L-alanine fermentation, glutamate degradation, and arginine biosynthesis capabilities were up-regulated in AMD patients. In another, slightly larger sample, the same authors showed that patients with nAMD (n = 57) were enriched in class *Negativicutes* compared to controls (n = 58).²⁸⁶ Moreover, the intestinal microbiomes were

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enriched in genes of the purine ribonucleosides degradation pathway in patients with nAMD. In a recent review, unpublished data described an enhanced abundance of the genera *Prevotella*, *Holdemanella*, and *Desulfovibrio* in patients with advanced AMD (n = 85) compared to healthy control subjects (n = 49).²⁸⁸ Further, a case-control study reported higher abundances of the genera *Bacteroides* and *Eubacterium_siraeum_group* in the AMD group (n = 30) compared with the control group (n = 17).²⁸⁹ Contrary to the results of the Swiss study, another recent Chinese case-control study observed a decreased *Firmicutes/Bacteroidetes* ratio in the fecal microbiota of patients with neovascular AMD (n = 30) compared to controls (n = 30). Integrating the publicly available datasets of the Swiss studies,^{285,286} they reported that the compositions of the gut microbiome differed significantly between the Chinese and Swiss cohorts with approximately 75 % bacterial species showing different abundance between the two cohorts ($P < 0.05$).

Thus, a distinct microbial signature linked to AMD may exist, however, the reported gut microbiota changes show discrepancies between the studies and do not overlap to an extent where specific shifts in composition can be confidently linked with AMD pathology.^{291–294}

The recent reviews by Zysset-Burri et al., Bringer et al. and Xiao et al. discuss the potential mechanism involved in gut microbial dysbiosis and AMD.^{291,292,295} Gut dysbiosis (due to Western diet, stress, metabolic syndrome, aging, etc.) may compromise the intestinal barrier, leading to increased intestinal permeability. This increased permeability allows antigens, lipopolysaccharides (LPS), pathogens, cytokines (among others IL-6, IL-1 β , TNF- α , and VEGF-A) and other proinflammatory substances to pass through intestinal barrier into the bloodstream, triggering systemic or local inflammation. Especially in individuals carrying the CFH risk variant, this could contribute to the development of AMD.²⁹⁵

The oral, nasal and pharyngeal microbiome

Concerning the oral microbiome, the potential associations between various periodontitis - related clinical symptoms and AMD has been explored,^{296–300} with the majority of studies describing a greater frequency of periodontitis -related symptoms among AMD cases compared to controls.^{297–299}

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A recent Canadian study reported on both the oral and nasal microbiota and its association with AMD.³⁰¹ In total, 8 nasal and 15 microbial communities showed a statistical difference between cases and controls. Concerning the nasal microbiome, *Burkholderiales* order, *Actinomycetaceae* family, *Gemella* genus, *Proteobacteria* family, *Streptococcus* species, *Actinomyces* species and *Veillonella* species were significantly higher in AMD cases (n = 13) as compared to controls (n = 5). Conversely, members of the *Clostridia* class were 4-fold higher in controls. In the oral mucosa, the *Rothia* genus, the *Propionibacteriales* family, the *Staphylococcus* species and the *Corynebacteriaceae* genus were among the communities with increased abundance in neovascular AMD cases compared to controls.

A study from Singapore reported an associations of the human pharyngeal microbiome and AMD.³⁰² Although the alpha diversity and overall composition of the microbiome were comparable between AMD patients (n = 245) and controls (n = 386), the AMD group exhibited a higher relative abundance of *Gemella* and *Streptococcus* compared to the controls.

Concerning the pathomechanism, secondary to changes in the oral, nasal and pharyngeal microbiota, local inflammation could enable the systemic spread of bacterial pathogens, microbial toxins (such as LPS), pro-inflammatory molecules or immune complexes to the eye, favoring the onset of AMD.

4.2.3.2 The human microbiome and retinal neurodegeneration / glaucoma

The gut microbiome

Concerning the link between the gut microbiome and retinal neurodegeneration / glaucoma, differences in certain taxa within the human gut microbial composition between POAG patients and controls have been recently described.^{237,238} Further, an association between POAG and mitochondrial DNA variants, associated with alterations in the gut microbiome, was described.^{303,304} Compared with controls, *Prevotellaceae* family, *Enterobacteriaceae* genus, and *Escherichia coli* species were reported with the strongest increase in abundance in POAG patients.²³⁷ Interestingly, also a positive correlation of *Streptococcus* in fecal samples with RNFL thickness was shown. Another study identified that the gut microbiome of glaucoma patients were rich in *Dysgonamonadaceae* family, along with a lower level of

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Barnesiellaceae family by metagenomic sequencing.²³⁸ Recently, a large Mendelian randomization (MR) analysis, including more than 100,000 POAG cases, was conducted to assess the causal links between gut microbiota taxa and POAG.²³⁵ They reported that with high statistical powers of the causal estimates *Odoribacter* genus, *Ruminiclostridium*9 genus and *Eubacterium rectale* species were associated with an increased risk of POAG. The authors further described that that genera *Oscilospira*, *Alloprevotella* and *Faecalibacterium* were positively associated with RNFL thickness, while *Ruminococcaceae* was negatively correlated to the RNFL thickness.

Regarding the underlying mechanisms, a mouse model revealed that mice raised without commensal microflora did not exhibit glaucomatous T-cell responses or the associated neurodegeneration.³⁰⁵ This suggests that glaucomatous neurodegeneration is partially driven by T cells that have been pre-sensitized through exposure to commensal microflora.

Another mouse study showed that reduction of these glaucoma-specific gut microbiome bacteria significantly reduced retinal ganglion cell loss by alleviating the activation of retinal microglia cells and overproduction of inflammatory cytokines.²³⁸ Further, they reported that in mice, microbiome transplantation from glaucoma patients significantly exacerbated retinal microglia activation and increased retinal inflammation.

The oral microbiome

The oral cavity is another reservoir of human microbiota, and oral microbiome dysbiosis has been also linked to glaucoma.²⁴⁰

Subjects with glaucoma were reported to show significantly higher oral bacterial loads and significantly fewer teeth.¹⁹⁰ A follow up study by the same authors found that *Streptococci* load is higher in the saliva of patients with POAG than in controls.³⁰⁵ Analysis of the oral microbiome using machine learning prediction models revealed significant enrichment of the genus *Faecalibacterium* in glaucoma patients.³⁰⁶

4.3 Perspectives

There are different aspects that emerge from the results of our analyses and the discussion and might be worth perusing in the future:

- 1) From a clinical point of view, the question if LPS levels in the blood could serve as a biomarker for AMD or glaucoma/RNFL thinning would be interesting to ascertain. Especially the time seems to play an important role here. Our blood draw was relatively far in the past, and could still show an association with early changes in AMD and baseline RNFL thickness. Therefore, it would be intriguing to now examine repeated measurements of LPS in relation to the progression of AMD and RNFL in order to shed more light on the potential temporal interplay of LPS with AMD and RNFL. Also, participants of the Alienor were over 73 years of age. It would be interesting to investigate these temporal relationships in a sample with a wider age range (ideally starting at age 45 or 50), in particular for studying early changes.
- 2) If the association of LPS and AMD/RNFL is confirmed in further studies, in particular in relation to the temporal aspect, it would be of interest to explore further the pathways that play a role in LPS-induced inflammation in retinal neurodegeneration, in particular a blockade of the TLR4 receptor. This is especially interesting against the background that once LPS has breached the blood-retina barrier, direct effects of LPS are known to lead to detrimental effects *in vivo*.
- 3) More generally, LPS has been shown to work strongly via oxidative damage. Thus, also the use of antioxidants and neuroprotective agents might be worthwhile to consider to protect mitochondrial function and help to prevent LPS-induced retinal damage.
- 4) In conjunction with the metabolic risk factors and the human microbiota, also the effect of diet seems to be linked indirectly (impairment of the gut due to Western diet and thus possibly more systemic release of LPS) and directly (postprandial elevation of LPS after fatty meals) with LPS. However, it may be very difficult to measure if an effect of diet did distinctly change the LPS influx and thus the influence of LPS on AMD or glaucoma progression. Still, looking at the body of evidence in favor for more prudent diet patterns like the Mediterranean diet, our results just simply add to the overall recommendation in favor of a more healthy and balanced nutrition.

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5) Since LPS is associated with metabolic conditions like obesity, insulin resistance, and cardiovascular diseases, targeting systemic inflammation and LPS exposure may not only reduce the risk of these conditions but also slow down or prevent the progression of AMD and retinal neurodegeneration. This is especially relevant given the aging population and the rising burden of AMD and glaucoma.

5 Conclusion

In summary, there are numerous hypotheses how LPS exerts its detrimental effect on the development and progression of AMD and retinal neurodegeneration, which might be interesting therapeutical targets for the future. Further, LPS might arise from different sources, including individual bacteria and the human microbiome; the gut microbiome representing the largest reservoir for Gram-negative bacteria in the human body.

Our results provide the first epidemiologic links between LPS and AMD, as well as LPS and retinal neurodegeneration. The work demonstrates the potential use of LPS as a biomarker for inflammation in eye diseases. However, more studies are needed to verify this link to further develop this potential biomarker in ocular aging.

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Appendix

Appendix 1

Protocol for the quantification of plasma esterified 3-hydroxy fatty acids

Plasma esterified 3-hydroxy fatty acids (3-OH FAs) were quantified as a proxy of total plasma LPS burden using liquid chromatography tandem mass spectrometry (LC-tandem MS), as previously described by Pais de Barros ¹³ et al. with modifications.

Two aliquots of human plasma (100 μ L) spiked with 4 pmol of internal standard (3-hydroxytridecanoic acid, 1 pmol/ μ L in ethanol) were used for unesterified and total 3-OH FAs quantification.

Unesterified (free) 3-OH FAs were extracted with ethanol (200 μ L), hydrogen chloride 50 mM (300 μ L), endotoxin free water (400 μ L), and ethyl-acetate/hexane 3:2 v:v (5 mL).

For total 3-OH FAs quantification, plasma was hydrolyzed with hydrochloride acid 8M (300 μ L) for 3 hours at 90°C. Fatty acids were further extracted with 600 μ L of endotoxin free water and ethyl-acetate/hexane 3:2 v:v (5 mL).

Samples were mixed for 5 minutes and then centrifuged for 5 min at 1700 g at room temperature. Organic phases (4.5 mL) were recovered using a Fluent 780 pipetting instrument equipped with a flexible 8 pipetting channel arm and DiTi LIHA 1000 μ L tips (TECAN, France).

Samples were then evaporated under vacuum using a Savant SC250 EXP Speedvac concentrator (ThermoScientific). Dried extracts were solubilized with ethanol (200 μ L) and transferred to injection vials with 300 μ L glass inserts, evaporated under vacuum and finally solubilized with ethanol (50 μ L).

Samples (3 μ L) were injected on a SBC18 2.1 x 50 mm, 1.8 μ m column maintained at 45°C at a flow rate of 0.4 ml/min. Mobile phases used were A: water containing formic acid (0.1 %) and ammonium formate (5 mM) – B : Acetonitrile/water 95/5 containing formic acid (0.1 %) and ammonium formate (5 mM).

Fatty acids were separated using an isocratic step at 45 % B for 0.5 min followed by a linear gradient up to 100 % B in 2.5 min and a final isocratic step at 100 % B for 5 min.

Appendix

Tandem MS detection was achieved with a 6490 triple quadrupole (Agilent Technologies) equipped with an ESI-JetStream source (Gas Temp 290°C, Gas Flow 19 L/min, Nebulizer 20 psi, Sheath Gas Heater 175°C, Sheath Gas Flow 12 L/min, Capillary 2000 V, Charging 2000 V, CA voltage 2 V). Acquisitions were performed in selected single reaction (SRM) negative mode. Deprotonated 3-OH FAs [M-H] were monitored based on the product ion of $m/z = 58.9$ Da which correspond to the CH₃COO fragment (collision energies ranging from 8 to 20 V depending on to length of the fatty acid chain). Human plasma calibration standards (100 µL) containing 0, 0.5, 1, 2, 4, 8, 16, 32 and 64 pmol of each of 3-OH FA from C10 to C18 were prepared and treated as samples. Area under curve for each 3-OH FA was determined and area to IS ratios were calculated. Linear calibration was used for calculations.

LPS-derived/esterified 3-OH FAs were calculated as the difference between total 3-OH FAs and unesterified 3-OH FAs. The resulting esterified 3-OH FA concentrations, expressed in picomolars per milliliter, are a proxy of total plasma LPS burden.

Appendix

Appendix 2

Could Infectious Agents Play a Role in the Onset of Age-related Macular Degeneration? A Scoping Review

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Topic: This scoping review aims to summarize the current state of knowledge on the potential involvement of infections in age-related macular degeneration (AMD).

Clinical relevance: Age-related macular degeneration is a multifactorial disease and the leading cause of vision loss among older adults in developed countries. Clarifying whether certain infections participate in its onset or progression seems essential, given the potential implications for treatment and prevention.

Methods: Using the PubMed database, we searched for articles in English, published until June 1, 2023, whose title and/or abstract contained terms related to AMD and infections. All types of study design, infectious agents, AMD diagnostic methods, and AMD stages were considered. Articles dealing with the oral and gut microbiota were not included but we provide a brief summary of high-quality literature reviews recently published on the subject.

Results: Two investigators independently screened the 868 articles obtained by our algorithm and the reference lists of selected studies. In total, 40 articles were included, among which 30 on human data, 9 animal studies, 6 in vitro experiments, and 1 hypothesis paper (sometimes with several data types in the same article). Of these, 27 studies were published after 2010, highlighting a growing interest in recent years. A wide range of infectious agents has been investigated, including various microbiota (nasal, pharyngeal), 8 bacteria, 6 viral species, and 1 yeast. Among them, most have been investigated anecdotally. Only *Chlamydia pneumoniae*, *Cytomegalovirus*, and hepatitis B virus received more attention with 17, 6, and 4 studies, respectively. Numerous potential pathophysiological mechanisms have been discussed, including (1) an indirect role of infectious agents (i.e. a role of infections located distant from the eye, mainly through their interactions with the immune system) and (2) a direct role of some infectious agents implying potential infection of various cells types within AMD-related tissues.

Conclusions: Overall, this review highlights the diversity of possible interactions between infectious agents and AMD and suggests avenues of research to enrich the data currently available, which provide an insufficient level of evidence to conclude whether or not infectious agents are involved in this pathology.

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Supplemental material available at www.ophtalmologyscience.org.

Age-related macular degeneration (AMD) is a progressive retinal disease¹ whose worldwide prevalence has been estimated at 8.7% in pooled global data of people between 45 and 85 years, which is expected to affect 288 million people by 2040.² Age-related macular degeneration is clinically classified into 3 stages according to the severity of fundus lesions.³ Early AMD rarely causes visual impairment and is described by medium-sized drusen, clinically visible deposits underneath the retinal pigment epithelium (RPE). These deposits are made of lipids (esterified and unesterified cholesterol), at least 129 different proteins (e.g. β -amyloid, apolipoproteins, complement regulation factors), metal ions

(zinc, iron), RPE cell debris, and immune cells.¹ Further disease progression to intermediate AMD is associated with larger drusen and pigmentary abnormalities of the macula. Advanced AMD includes geographic atrophy (GA) and neovascular AMD: GA is characterized by gradually expanding retinal thinning due to loss of photoreceptors, RPE cells, and choroidal capillaries.¹ Neovascular AMD corresponds to an invasion of new choroidal blood vessels (choroidal neovascularization [CNV]) into the subretinal and/or sub-RPE space that can lead to sight-threatening exudation of fluid and/or blood. Both GA and neovascular AMD result in substantial visual

decline in most patients and can also coexist in 1 eye. Therapeutic options remain limited. For the treatment of neovascular AMD, intravitreal therapy using anti-VEGF has proven its ability to reduce the incidence of AMD-related legal blindness. However, life-long injections are often required, which cannot always prevent a slow deterioration of vision.^{4,5} For the treatment of GA, pegcetacoplan, a complement C3 inhibitor, was approved in 2023 in the United States, following the observation of reduced GA growth compared with sham treatment.^{6,7} However, to date, no effective therapies for early or intermediate AMD exist and none of the approved therapies for advanced AMD are curative.

The pathophysiology of AMD is complex and the underlying mechanisms are not yet fully understood. Mitochondrial dysfunction together with oxidative stress and defects in autophagy have been described to contribute to AMD.⁸ A significant inflammatory component involving the complement system and various types of immune cells (microglia, macrophages, dendritic cells) have been also implicated in AMD.⁹ Large epidemiological studies have also revealed several risk factors of AMD including age, genetics,¹⁰ and some environmental factors.^{1,11} The genetic risk of AMD is considered to be linked to multiple genetic loci of small to modest effect contributing to the disease. However, 2 loci have been consistently strongly linked to advanced AMD in genome-wide association studies: Complement factor H (CFH) gene at the 1q31 susceptibility locus, where the C allele of CFH Y402H is recognized as a risk factor for AMD, and the 10q26 ARMS2/HTRA1.^{10,12–17} Additionally, large genome-wide association studies by an international consortium have identified 34 loci associated with AMD at a genome-wide significance level.¹⁰ Although certain risk factors for AMD such as cigarette smoking and dietary intake of antioxidants and fatty acids are widely accepted, others are more controversial, such as sunlight exposure, cardiovascular risk factors, alcohol consumption, etc.

In recent years, an infectious hypothesis in the pathogenesis of AMD has emerged, suggesting a link between various infectious agents and AMD. This hypothesis stems mainly (1) from the implication of low-grade inflammation in AMD and (2) from the knowledge that infection can exacerbate the inflammatory response. In the fields of infectious diseases and immunology, there is a growing body of research linking chronic low-level infections to diseases that are not typically thought of as infectious. In particular, chronic bacterial infections (e.g., *Chlamydia pneumoniae* [*C. pneumoniae*], *Helicobacter pylori* [*H. pylori*]) have been discussed to be linked to conditions like atherosclerosis and cancer.^{18–20} This has led to a greater awareness that chronic or latent infections could still have long-term consequences for tissue health, including those of the eye. In neurodegenerative diseases like Alzheimer's disease and Parkinson's disease, several infections have also been suggested to play a role in triggering or accelerating neurodegeneration.^{21,22} This idea has been extended to the retina, which is considered part of the central nervous system. In

order to assess the current state of knowledge on the diverse pathogens studied (including bacteria, yeasts, and viruses), we decided to conduct this scoping review. Our objectives were to investigate which pathogens had been studied, using which methodology, and which underlying pathophysiological mechanisms were suspected. Ultimately, we wanted to assess the current level of evidence for the infectious hypothesis in AMD and identify potential avenues for further research.

Methods

Eligibility Criteria for Considering Studies for This Review

Following the PRISMA scoping review guidelines,²³ we conducted a scoping review aiming to identify articles in English assessing the potential involvement of infectious agents in AMD.

- In vitro, animal, and human studies (including postmortem studies) as well as hypothesis papers were considered. Literature reviews and editorials were excluded.
- All types of conventional infectious agents were considered including bacteria, viruses, fungi (whatever the diagnostic method) as well as various proxies (e.g., pathogen free environment versus conventional open environment). Nevertheless, given that recent high-quality literature reviews already existed on oral and gut microbiota,^{24–27} we decided not to include the related articles in this review. In addition, an article dealing with the impact of the recent severe acute respiratory syndrome coronavirus type 2 (SARS-COV2) pandemic on AMD was also excluded because it appeared that our algorithm did not allow for the proper identification of studies involving SARS-COV2 in AMD. Indeed, existing studies investigate the consequences of SARS-COV2 infection on all ocular structures (not specifically AMD) and, consequently, AMD-related terms were not systematically present in their title and/or abstract. Finally, our algorithm was not designed to identify studies investigating only certain bacterial components (e.g., lipopolysaccharide [LPS]), frequently used to study the impact of inflammation; we therefore excluded these studies as well.
- In human studies, all AMD stages were considered and AMD diagnoses could be based on clinical or medico-administrative data. In in vitro and animal studies, all types of AMD-related lesions (e.g., drusen, CNV, etc) were considered.

Search Methods for Identifying Studies

We searched the PubMed database for articles in English published up to June 1, 2023, without any temporal or geographical limitations. Using a search algorithm to find articles whose title and/or abstract contain a combination of terms related to (1) AMD and (2) infectious agents

(details in Appendix 1 available at <https://www.ophtalmologyscience.org>), we identified 868 articles related to our topic of interest. The list of records identified was exported into the reference management software Zotero 7 (Corporation for Digital Scholarship) and into an Excel table (Microsoft Corporation) to assess eligibility criteria. Subsequently, we also screened the reference lists of selected studies.

No institutional board review/ethics committee approval was required for this scoping review.

Study Selection

After removing duplicates ($n = 2$), a retracted article ($n = 1$), and an editorial ($n = 1$), 2 investigators (L.P. and L.M.) independently screened the 864 remaining references and then assessed 89 reports for eligibility (flow chart in Fig 1). To note, 2 articles in Russian^{28,29} could not be included, although they dealt with our subject of interest. Three additional records were identified from citation searching using the reference lists of selected studies and assessed for eligibility. Discrepancies were resolved through discussion between the investigators. In total, 40 articles were included in our review (see the list of included articles in Table S1 available at <https://www.ophtalmologyscience.org>).

Data Collection

From the selected articles, we systematically extracted the following items in calibrated Excel tables.

- For human studies, we extracted the following: first author, title, journal, year of publication, infectious agent(s) investigated, data source (including location, type of study, baseline, or study period if available), study design, participants (including number and main inclusion/exclusion criteria), diagnostic methods for both infection and AMD, statistical methods, and main results.

- For experimental studies, we extracted the following: first author, title, journal, year of publication, infectious agent(s) investigated, type of study, cell line studied or used animal models, exposure protocol for the infectious agent in question, main outcomes, and results.

L.P. extracted data for studies involving bacteria or fungi and L.M. for viruses or infectious proxies. The extraction was then checked by the second investigator.

To note, for the classification of AMD, several internationally accepted classifications exist.^{8–10} To ensure comparability and consistency within this article, we used the widely accepted Beckman classification and harmonized the terminology of included studies. Of note, according to the Beckman classification, the term dry AMD is only used for GA. Therefore, the term nonexudative AMD was used to summarize early AMD, intermediate AMD, and GA. We are aware that in light of new insights learned from OCT angiography (OCTA), also the term nonexudative may be not entirely unambiguous due to the existence of non-exudative CNV.¹¹ However, most of the included studies were performed in the pre-OCTA era, and none of the included studies applied OCTA, indicating that all studies included in this scoping review refer to exudative CNV when using the term CNV.

Data Synthesis

The results of each of the 40 studies are detailed in Tables S2 and S3 (available at <https://www.ophtalmologyscience.org>) and summarized by pathogen type in the Results section below. An overview of the studies is also presented in Table 4. Moreover, in order to present a relatively complete overview of the topic, a brief summary of previously published literature reviews on the oral and gut microbiota is also presented at the end of the Results section.

Potential pathophysiological mechanisms are discussed (1) pathogen by pathogen, according to the elements evoked in each of the articles in the Results section and (2) more globally in the Discussion section.

Results

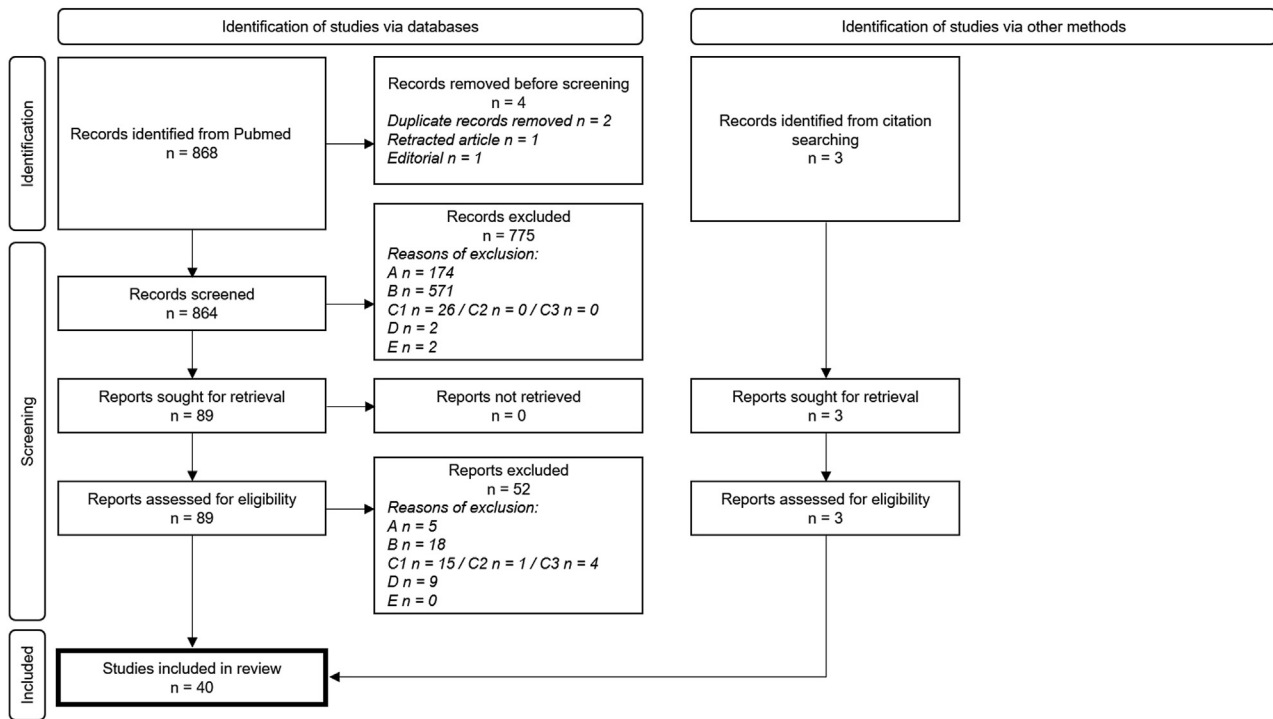
After screening 868 articles, 89 were assessed in full for eligibility (detailed flow chart in Fig 1). In total, we included 37 original articles or letters in English dealing with the potential impact of infectious agents in AMD. Reviewing the reference lists of all selected articles, we identified 3 additional articles that were not captured by our research algorithm (one letter to the editor on varicella-zoster virus [VZV]³⁰ and 2 studies on *C. pneumoniae*^{31,32}). Finally, 40 articles were included in our review. The results of these studies are detailed in Tables S2 and S3 (available at <https://www.ophtalmologyscience.org>) and briefly presented below by pathogen type (see also Table 4 for an overview).

Briefly, the results described in these 40 studies were obtained from human data ($n = 30$), animal ($n = 9$) or in vitro ($n = 6$) experiments (sometimes with several data types in the same article), and one hypothesis paper. The studies focused on either bacteria ($n = 21$), fungi ($n = 1$), viruses ($n = 15$), or other biomarkers of infections ($n = 4$).

Studies Included in Our Review

Chlamydia Pneumoniae. We identified a total of 17 original studies on *C. pneumoniae* and AMD.^{31–47} Among them, 12 studies investigated the potential associations between *C. pneumoniae* serological status and either AMD ($n = 10$) or its progression ($n = 2$). Four studies searched for the presence of *C. pneumoniae* within human eye tissues and 3 were experimental studies (in vitro and/or on animal models). Of note, one study included both serological and histopathological data, and another study included both histopathological and experimental data.

Five studies explored the potential association between seropositivity to *C. pneumoniae* and the prevalence of AMD.^{31,41–43,46} Among these studies, 3 were case-control studies,^{41,42,46} one a nested case-control study,³¹ and one was a cross-sectional study.⁴³ Of note, in the nested case-control study, the authors also investigated the impact of *C. pneumoniae* infection on the incidence or progression of AMD after 10 years. None of the case-control studies found a significant association between *C. pneumoniae* seropositivity and AMD. Associations were also nonsignificant in studies analyzing early AMD, GA, or neovascular AMD



Reasons of exclusion: A = Doesn't deal with AMD; B = Doesn't deal with a potential link with infectious disease; C1 = Deals only with oral or gut microbiota; C2 = Deals only with SARS-COV2; C3 = Deals only with lipopolysaccharide; D = Literature review; E = Not in English

Figure 1. Flow chart illustrating the article identification and screening process. AMD = age-related macular degeneration; SARS-COV2 = severe acute respiratory syndrome coronavirus type 2.

separately.^{31,42,46} Conversely, the cross-sectional study by Nakata et al (which benefited from a larger sample size of 971 subjects with large drusen and 3209 without) reported a significant association between *C. pneumoniae* seropositivity and intermediate AMD despite relatively small differences in seroprevalence (56.4% in subjects with intermediate AMD vs. 51.7% in those without; $P = 0.01$). One study⁴⁶ also investigated potential interactions with genetic risk factors: it highlighted that, among carriers of CFH Y402 risk *C. alle*, seropositivity for *C. pneumoniae* differed slightly between early and advanced AMD ($P = 0.09$, 60.0% of infected subjects in early AMD vs. 75.7% in advanced AMD) whereas there was no difference between controls and pooled AMD cases (70.1% and 72.2%, respectively).

Eight studies explored the potential association between the levels of anti *C. pneumoniae* immunoglobulin G (IgG) (which may reflect more intense exposure to *C. pneumoniae* [i.e. higher concentrations of bacteria as well as chronic or recurrent infections]) and the prevalence of AMD ($n = 6$) or its progression ($n = 2$). Among these, 4 case-control studies^{33,35,36,42} found mixed results. The first 2 studies highlighted significantly increased levels of anti *C. pneumoniae* IgG in subjects with any AMD³³ or neovascular AMD³⁵ compared with controls. The next 2 studies^{36,42} found no statistically significant association between these levels of anti *C. pneumoniae* IgG and AMD, studying nonexudative AMD (i.e., early AMD, intermediate AMD, or GA), neovascular AMD, and controls separately. All these 4 studies suffered from small

study samples of less than 100 cases and limited adjustment. The 2 remaining studies on anti *C. pneumoniae* IgG and the prevalence of AMD had larger study samples with wide adjustment.^{38,43} The cross-sectional study by Nakata et al revealed a significant association between anti *C. pneumoniae* IgG levels and the prevalence of intermediate AMD, indicated by the presence of drusen $\geq 125 \mu\text{m}$ (adjusted odds ratio [aOR] = 1.020 95% confidence interval [95%CI] [1.001–1.040]; $P < 0.05$). The nested case-control study by Robman et al found no significant association between levels of anti *C. pneumoniae* IgG and any prevalent AMD ($n = 197$ cases of AMD, aOR = 1.02 95%CI [0.66–1.56] comparing the third tertile to the first tertile of IgG), early AMD ($n = 159$ cases, aOR = 0.97 95%CI [0.61–1.53]), or advanced AMD ($n = 38$ cases, aOR = 1.23 95%CI [0.52–2.92]). Nevertheless, given the limited number of advanced AMD cases, a differential impact of the infection depending on the AMD stage remains to be investigated. Finally, 2 studies performed within the prospective “Cardiovascular Health and Age-related Maculopathy Study” explored the association between levels of IgG and the progression of AMD (using a 6-level severity scale and 3 different definitions of grading) in a cohort of participants with early AMD.^{37,40} They highlighted that subjects in the second and the third tertile of anti *C. pneumoniae* IgG titers had a significantly increased risk of 7-year AMD progression compared with those in the first tertile (aOR = 2.10 95%CI [1.00–4.50] and aOR = 2.58 95%CI [1.24–5.41], respectively).

Table 4. Brief Overview of the Main Results of the 40 Studies Identified

Human Studies		Studies on Animal, In	
Chlamydia pneumoniae (Cp)	Association between Cp serostatus and AMD (n = 11): Seropositivity to Cp: [X][X]³¹ [X]⁴¹ [X]⁴² [X]⁴⁶ [X]⁴³ Levels of anti-Cp antibodies: [X]³³ [X]³⁵ [X]³⁶ [X]³⁷ [X][X]³⁸ [X]⁴⁰ [X]⁴² [X]⁴³ Search for Cp in human tissues (n = 4): PCR study using peripheral blood cells: [X]⁴⁷ PCR/IHC studies using ocular tissues: [X]⁴⁷ [X]³⁴ [X]³⁹ [X]³²	In vitro or animal studies (n = 3): Human Cp-infected macrophages and RPE production of VEGF and inflammatory Murine RPE cells in contact with Cp and VEGF. Intravitreal injection of sizes of laser-induced CNV compared secretion of IL-6 and VEGF in the mice intraperitoneally infected with Cp had increased size of laser-induced CNV exposure to low doses of LPS induced reprogramming of retina-resident microinflammatory but enhanced angiogenesis	
Nasal or pharyngeal microbiota	Investigation of the microbiota using 16s rRNA gene sequencing (n = 2): Nasopharynx microbiota: [X][X]³¹ [X]⁵¹ Nasal microbiota: [X]⁵²		
Other bacteria and yeasts	Association between certain bacteria and AMD (n = 4): <i>Chlamydia trachomatis</i>: [X]⁴⁶ [X]³³ <i>Chlamydia psittaci</i>: [X]⁴⁶ <i>Escherichia coli</i>: [X]³³ <i>Mycoplasma pneumoniae</i>: [X][X]⁴² <i>Helicobacter pylori</i>: [X][X]³⁶ Case report (n = 1) suggesting an impact of <i>M. chelonae</i> on progression to neovascular AMD: [X]⁵³ Search for <i>B. megaterium</i> in soft drusen (n = 1): [X]⁵⁴	Animal studies (n = 2): Drusen-like pathology after injection of <i>H. pylori</i> in mice Systemic exposure to <i>Candida albicans</i> led to increased size of laser-induced CNV in mice	
HIV	Association between HIV and AMD (n = 2) using: Clinical diagnosis: [X]⁵⁶ [X]⁵⁷		
Hepatitis B virus (HBV)	Association between HBV and AMD (n = 4) using: Serostatus: [X]⁶⁰ [X]⁶¹ Medicoadministrative data: [X]⁶² [X]⁶³	In vitro study (n = 1): HBV could sensitize RPE cells to UV or through downregulation of DNA repair	
Hepatitis C virus (HCV)	Association between HCV and AMD (n = 4) using: Serostatus: [X]⁶⁰ Serostatus and confection in patients with HIV: [X]⁵⁶ [X]⁵⁷ Medico-administrative data: [X]⁷⁶		
Varicella-zoster virus (VZV)	Association between VZV and AMD (n = 1) using: Diagnosis of herpes zoster ophthalmicus in medico-administrative data: [X]⁵⁰		

Table 4. (Continued.)

Human Studies		Studies on Animal, In
Cytomegalovirus (CMV)	Association between CMV and AMD (n = 1) using: Levels of anti-CMV antibodies: [O] ⁹⁶ [●] ⁹⁷ Anatomopathological study (n = 1) searching for CMV DNA in posterior eye cups of human cadavers without AMD: ▲ ⁹⁸	In vitro or animal studies (n = 5): Macrophages infected with murine CMV levels of proangiogenic factors, consist Systemic murine CMV infection led to lesions related to the activation of CMV-infected macrophages toward a Following systemic infection, murine CMV infection within the choroid and in few infection was associated with increase proangiogenic factors as well as with re ^{90–92}
Human herpes virus 6A (HHV-6A)	Hypothesis paper (n = 1) on HHV-6A and AMD: ▲ ⁹⁴	Systemic murine CMV infection led to a microglial cells within the subretinal s
Other markers of infection	History of lung infection ◆ ⁹⁷ Specific microbial signature in the aqueous humor of patients with AMD ■ ¹⁰⁰	In vitro or animal studies (n = 2): Complement factor H (CFH) knock out open environment had higher number higher levels of proinflammatory cytok reduced number of photoreceptors, co exposed to a pathogen free environme Stimulation of human RPE cells with vir induced the expression of the angiogen cytokines, and complement proteins. C bacterial DNA analog had a lesser imp
Total*	30	13

Study design and conclusions: □ Case-control ○ Cross-sectional ◇ Cohort or nested case-control ▲ Other study design. Filled or empty shapes for infections and AMD, respectively.

AMD stages investigated: Black: all stages of AMD; Light blue: early AMD; Dark blue: progression from early AMD; Purple: intermediate AMD; Green: nonexudative geographic atrophy; Red: neovascular AMD.

When, in the same study, results are available for different stages of AMD or for different designs, they are presented in square brackets.

AMD = age-related macular degeneration; B. megaterium = Bacillus megaterium; CMV = Cytomegalovirus; CNV = choroidal neovascularization; DNA = deoxyribonucleic acid; IHC = immunohistochemistry; IL = interleukin; LPS = lipopolysaccharide; M. chelonae = Mycobacterium chelonae; PCR = polymerase chain reaction; rRNA = ribosomal ribonucleic acid; VZV = varicella-zoster virus.

*Some studies concern several pathogens and use several study methods.

Moreover, they hypothesized a potential interaction between infection by *C. pneumoniae* and the CC risk genotype of the CFH gene. In multivariate models, although the CC genotype was associated with an increased risk of AMD progression (aOR = 2.43 95%CI [1.07–5.49], $P = 0.03$, compared to the TT genotype), having both the CC genotype and being within the upper tertile of antibodies to *C. pneumoniae* was associated with a greater risk of AMD progression (aOR = 11.8 95%CI [2.1–65.8], $P = 0.005$, compared to TT genotype within the lowest tertile).

Regarding the underlying mechanisms, several hypotheses have been discussed. Firstly, *C. pneumoniae* is an obligate intracellular bacterium which can infect the respiratory tract and establish persistent infection in humans and subsequent chronic inflammation. Such chronic systemic inflammation has been proposed to have an impact at sites distant from the infection and could therefore contribute to diseases with an inflammatory component such as AMD. To note, a role of *C. pneumoniae* has been suggested in several other inflammatory diseases (including atherosclerosis) but remains controversial.^{18,48} Exposure to peripheral immune stimuli (such as *C. pneumoniae* or the endotoxin LPS, which is part of its outer membrane) has also been suggested to provoke a long-term reprogramming of immune cells at distant sites (including retina-resident microglia), which could have detrimental effects upon subsequent immune stimuli, potentially contributing to the onset of AMD.⁴⁵ Secondly, infection of macrophages by *C. pneumoniae* allows dissemination of *C. pneumoniae* to various organs including ocular tissues and especially the choroid (given the particular attraction of *C. pneumoniae* for vascular tissue). Subsequently, *C. pneumoniae* may infect various cell types, including RPE cells (which are the first line of defense against external pathogens by maintaining of the blood–retina barrier) or retinal microglial cells. Once in the eye, *C. pneumoniae* could promote AMD through various mechanisms including local chronic secretion of inflammatory cytokines and chemokines (and subsequent recruitment of immune cells⁴⁹), the secretion of proangiogenic factors or an induction of lipoprotein oxidation, dysregulation of cellular lipid metabolism, and dysregulation of apoptotic pathways, all mechanisms which have been implicated in AMD pathogenesis.^{33,34} Because the most common trigger of the alternative pathway are microbial in nature, *C. pneumoniae* could also lead to uncontrolled complement activation, in particular in subjects carrying a risk variant of the CFH gene.^{38,50}

Several studies have provided clues as to the mechanisms potentially involved, analyzing the presence of *C. pneumoniae* in human tissues and/or blood. In 2009, one study⁴⁷ analyzed the presence of *C. pneumoniae* DNA in peripheral blood cells by polymerase chain reaction (PCR), highlighting a higher prevalence of infection in subjects with advanced AMD ($n = 148$; 20.3%) than in controls ($n = 162$; 10.5%; $P = 0.02$). Conversely, PCR analyses of microdissected macular cells of paraffin-embedded ocular slides showed the presence of *C. pneumoniae* in only a small number of samples: 2 out of 59 advanced AMD samples (GA or neovascular) (3.4%)

and one out of 16 (6.3%) age-matched control samples (the difference was not statistically significant). Three studies analyzed the presence of *C. pneumoniae* in CNV, using surgically excised CNV specimens of patients with AMD. These CNV samples were excised in the pre-anti-VEGF era, when surgical removal of CNV used to be a valid therapy option. In 2005,³⁴ one study reported *C. pneumoniae* in 4 out of 9 (44.4%) and 2 out of 9 (22.2%) AMD CNV paraffin-embedded specimens, using immunohistochemistry (IHC) and PCR, respectively. Conversely, *C. pneumoniae* was not found in any of the 21 control samples (including in particular 5 non-AMD CNV specimens and 16 non-AMD eyes). In 2006, however, one study³⁹ using 13 AMD CNV frozen specimens did neither detect *C. pneumoniae* DNA by PCR, nor any other bacterial DNA using a broad range bacterial 16S ribosomal DNA PCR. In 2013, one study using IHC³² detected *C. pneumoniae* in 17 out of 25 (68.0%) AMD CNV paraffin-embedded specimens. Notably, macrophages were also visualized in 19 specimens including 16 positive to *C. pneumoniae* using IHC. These discrepancies between studies may be explained by the use of different methodologies (PCR vs. IHC) and pretreatments of analyzed tissues. To note, the detection of *C. pneumoniae* might have been underestimated (1) in PCR studies using formalin-fixed tissues (as formalin degrades DNA)³⁴ and (2) due to a patchy distribution of the infection.^{32,34} One of the studies using IHC showed that on average only 6% (varying from 1% to 18%) of the cells were infected by *C. pneumoniae*, indicating a strong influence of sampling on the results.³² However, neither the absence of *C. pneumoniae* excludes its pathological role (i.e., the "hit and run hypothesis" suggests a secondary elimination of *C. pneumoniae* after damage has occurred), nor does its presence confirm its causal role (i.e., the "innocent bystander hypothesis" suggests that pre-existing inflammation could promote the recruitment of *C. pneumoniae*-infected monocytes to already damaged ocular tissues).

Several in vitro and animal studies have also investigated some of the potential underlying mechanisms. In 2005, *C. pneumoniae* was shown to infect both human macrophages and RPE cells in vitro. Moreover, it was reported that the infection increased the production of VEGF by human macrophages and that of interleukin (IL)-8 and monocyte chemoattractant protein-1, both inflammatory chemokines implicated in the pathogenesis of AMD, by RPE cells.³⁴ In 2010,⁴⁴ a study showed that the inoculation of *C. pneumoniae* antigen to primary-cultured murine RPE cells led to a toll-like receptor 2 dependent secretion of IL-6 (multifunctional inflammatory cytokine promoting both angiogenesis and host defense against pathogens) and VEGF. Notably, stimulation with LPSs also led to the secretion of IL-6, VEGF, and tumor necrosis factor (TNF) α by RPE cells. Consistently, using C57BL/6 mice, intravitreal injection of the *C. pneumoniae* antigen was shown to increase the size of laser-induced CNV and to increase secretion of IL-6 and VEGF in the intraocular fluid compared to controls. The use of different knockout mouse models also confirmed the toll-like receptor 2-dependent mechanism. In 2023⁴⁵, using intraperitoneally infected

C57BL/6J mice, Hata et al confirmed the larger size of laser-induced CNV in infected mice compared to controls. Subsequently, they investigated the impact of *C. pneumoniae* infection through exposure to LPS showing that laser treatment performed 4 weeks after exposure to low doses of LPS led to more pronounced CNV in LPS-exposed mice than in controls. Further experiments led them to conclude that prior peripheral exposure to low doses of LPS induced a long-term epigenetic reprogramming of retina-resident microglia and subsequent reduced inflammatory but enhanced angiogenic phenotype, which may contribute to the development of CNV lesions. The apparent discrepancy between proinflammatory and anti-inflammatory properties of *C. pneumoniae* may be explained by differences in experimental conditions (in vitro, in vivo, the timing, the intensity, and the location of exposure).

Nasal and Pharyngeal Microbiota. In 2018, a case-control study from Singapore investigated the differences of microbial colonization of the human nasopharynx between AMD patients (both early and advanced AMD) and controls, using 16s ribosomal RNA gene sequencing.⁵¹ Although the alpha-diversity (bacterial variation within a single sample) indices and the global composition of the microbiome were similar between AMD patients (n = 245) and controls (n = 386), subtle differences in relative abundance for some bacterial genera were highlighted. Generalized Linear Mixed Model analysis revealed that AMD cases had increased relative abundance of *Gemella* (6.0% vs. 4.0%, adjusted $P = 0.007$) and *Streptococcus* (23.4% vs. 18.6%, adjusted $P = 0.002$) and decreased relative abundance of *Prevotella* (12.7% vs. 19.3%, adjusted $P = 6.95E-5$) and *Leptotrichia* (0.8% vs. 1.4%, adjusted $P = 0.007$) compared with controls. Comparing advanced AMD to controls produced similar results while early AMD did not differ from controls, suggesting potential dysbiosis in advanced stages of the disease. Analyzing the subset of patients >60 years old revealed significantly different relative abundances for the *Prevotella*, *Leptotrichia*, and *Streptococcus* genera in AMD cases compared to controls.

In 2020, a small study from Canada⁵² published data on nasal microbiota and its association with neovascular AMD, also using 16s ribosomal RNA gene sequencing. Some bacterial communities had an increased relative abundance in AMD cases (n = 13) compared to controls (n = 5) including the *Burkholderiales* order (sevenfold higher; $P = 3.29E-05$), the *Actinomycetaceae* family (sixfold higher; $P = 3.73E-06$), the *Gemella* genus (fivefold higher; $P = 0.0002$), the *Proteobacteria* family (fourfold higher; $P = 0.004$), the *Actinomyces* species (threefold higher; $P = 0.002$), the *Streptococcus* species (threefold higher; $P = 0.01$), and the *Veillonella* species (twofold higher; $P = 0.005$). Conversely, members of the *Clostridia* class were fourfold higher in controls ($P = 0.007$).

Regarding potential underlying mechanisms, the authors hypothesize that changes in the composition of nasal and pharyngeal microbiota could lead to local inflammation and increased mucosal permeability. Subsequently, this could

facilitate transmission of bacteria or the dissemination of proinflammatory products to distant sites (such as the eye) inducing inflammation.

Other Bacteria and Fungi. *Mycobacterium Chelonae*. In 2016, a rare case of subacute bilateral transformation from nonexudative to neovascular AMD associated with bilateral choroiditis was described in a patient of about 70 years of age.⁵³ The authors hypothesized a possible involvement of *Mycobacterium chelonae* (*M. chelonae*), given (1) a recent episode of lung infection due to *M. chelonae* and (2) a history of autoimmune disease (which can favor the hematogenous dissemination of the bacteria). They suggested that mycobacterial dissemination may have induced bilateral choroiditis, triggering a local proangiogenic response, which may have resulted in progression to neovascular AMD.

Bacillus Megaterium. In a review from 2018⁵⁴, the authors mentioned unpublished data highlighting (1) an enrichment with *Bacillus megaterium* (*B. megaterium*) in soft drusen in patients with AMD and (2) an induction of a drusen-like pathology after subretinal injection of *B. megaterium* in a nonhuman primate model. No mechanistic details were reported, and, to our knowledge, the complete data are still unpublished.

Other Bacteria. Several studies mainly focusing on *C. pneumoniae* also reported results on other types of bacteria. A case-control study³³ found similar levels of IgG directed against antigens of *Chlamydia trachomatis* (*C. trachomatis*) and *Escherichia coli* (*E. coli*) comparing 25 AMD cases and 18 controls. Another case-control study⁴² found no significant differences between the levels of IgG directed against *Mycoplasma pneumoniae* (*M. pneumoniae*) in subjects with neovascular AMD (n = 20), nonexudative AMD (n = 20), and age- and sex-matched controls (n = 20). In a third case-control study,⁴⁶ including 199 patients with AMD and 100 controls, seropositivity to *C. trachomatis* or *Chlamydia psittaci* (*C. psittaci*) was not significantly associated with AMD. Finally, Miller et al³⁶ found similar levels of IgG directed against *H. pylori* comparing neovascular AMD (n = 47), nonexudative AMD (n = 36), and controls (n = 67).

Candida Albicans. In 2014, using C57BL/6J mice injected intravenously with a sublethal dose of *Candida albicans* (*C. albicans*), the authors highlighted that a systemic infection with *C. albicans* can cause (1) an increase in the number of microglial cells in the retina, (2) microglia activation, and (3) microglial relocation within the retinal layers.⁵⁵ Regarding the underlying mechanisms, *C. albicans* was not detected in retinal tissue. Thus, the authors suggested that microglia activation was because of either a high cytokine production secondary to systemic infection or to a direct interaction between microglia and soluble fungal ligands. The authors hypothesized that excessive or prolonged activation of microglia may subsequently lead to chronic retinal inflammation and subsequent retinal damage.

Human Immunodeficiency Virus. Using data from the American "Longitudinal Study of the Ocular Complications of AIDS" (LSOCA), Jabs et al⁵⁶ found a prevalence of 9.9%

of intermediate-stage AMD (graded by retinal photographs at inclusion) among 1825 patients with AIDS and without ocular opportunistic infections. The comparison to the previously published cohort of 2810 human immunodeficiency virus (HIV)-uninfected subjects of the Beaver Dam Offspring Study highlighted a threefold increase in crude prevalence of AMD for AIDS patients (9.9% vs. 3.3%) and a fourfold increase after adjustment for age and sex. Subsequently, the authors also compared the incidence of intermediate-stage AMD at the 5- and 10-year follow-ups in the LSOCA study (730 and 379 participants, respectively) to that estimated among approximately 3685 HIV-uninfected participants of the Multi-Ethnic Study of Atherosclerosis (MESA).⁵⁷ Patients with AIDS had an increased risk of intermediate-stage AMD (relative risk = 1.75 95%CI [1.16–2.64]; $P = 0.008$ after adjustment for ethnicity and sex) although HIV-uninfected subjects of the MESA cohort were older than those included in the LSOCA cohort (61 ± 9 vs. 44 ± 8 years). Thus, the authors hypothesized that the increased risk of AMD seen in patients with AIDS was linked to the impact of HIV on the immune system. Indeed, HIV infection has been associated to chronic systemic inflammation, chronic immune activation, and a certain degree of immunosenescence,^{58,59} components that could influence the risk of AMD. Moreover, as mentioned by the authors, their results could also be explained by selection bias. Because all participants in the LSOCA study were enrolled at AIDS ophthalmology clinics, they were more likely to have ocular diseases than participants in the Beaver Dam Offspring Study and the MESA study, which were population-based studies.

Hepatitis B Virus. In 2008, using data from a South Korean cross-sectional study⁶⁰ including 9530 participants >40 years of age, Roh et al evaluated potential risk factors for AMD. Age-related macular degeneration was diagnosed by fundus photography in 235 (2.5%) subjects, of which 215 had early AMD. Carriers of hepatitis B surface antigen (measured in the serum of patients and reflecting present hepatitis B virus [HBV] infection) had a twofold increased risk of AMD compared with uninfected subjects (aOR = 2.56 95%CI [1.48–4.42] after adjusting for known risk factors of AMD including sociodemographic status). Subsequently, another South Korean study⁶¹ including 14 352 participants above the age of 40 years found similar results in 2014: hepatitis B surface antigen carriers had a twofold increased risk of early AMD compared with uninfected subjects (aOR = 1.98 95%CI [1.38–2.85], $P < 0.001$). Although no significant association was found between HBV infection and advanced AMD (aOR = 1.49 95%CI [0.51–4.32], $P = 0.47$, after adjustment for age, sex, and smoking status), this analysis appears to suffer from low statistical power (only 5 infected subjects with advanced AMD).

More recently, 2 prospective studies^{62,63} based on the Taiwanese Longitudinal Health Insurance Database 2000 have also investigated associations between HBV and either macular degeneration or AMD. Unlike the 2 previous studies, which benefited from systematic screening, diagnoses of HBV and macular degeneration

were based on information available in the health insurance database leading to a risk of underdiagnosis for both pathologies. Nevertheless, this limitation is partly counterbalanced by the very large size of the samples. The first study included 39 796 subjects with HBV (whether acute or chronic) over the age of 20 and 159 184 age-matched controls and highlighted that infected subjects had an increased risk of macular degeneration (adjusted hazard ratio [aHR] = 1.31 95%CI [1.17–1.46] after adjustment for age, sex, and some comorbidities but not tobacco consumption). After dividing the study population into 3 age groups (≤ 34 , 35–49, and ≥ 50 years), this association remained statistically significant only in subjects >50 years of age (aHR = 1.24 95%CI [1.10–1.40]). Subsequently, a second analysis of these data was carried out focusing on subjects with chronic HBV infection. Using data of 17 796 subjects over the age of 40 with chronic HBV infection and 71 184 age and sex-matched controls, an increased risk of AMD (aHR = 1.41 95%CI [1.23–1.63], $P < 0.001$, after adjustment for age, sex, socioeconomic factors, several comorbidities but not tobacco consumption) was observed. Associations remained statistically significant when focusing on nonexudative AMD (aHR = 1.41 95%CI [1.19–1.65], $P < 0.001$), neovascular AMD (aHR = 1.43 95%CI [1.08–1.89], $P = 0.01$), and the progression from nonexudative to neovascular AMD (aHR = 1.74 95%CI [1.01–2.99], $P = 0.01$). Although these analyses appear to confirm the results obtained in the South Korean studies, it should be noted that a follow-up bias cannot be excluded (i.e. subjects with HBV infection are probably more frequently followed ophthalmologically because of HBV, its treatment or to a higher frequency of related comorbidities), and therefore have a greater likelihood of being diagnosed with AMD.

Regarding the underlying pathophysiological mechanism, several hypotheses have been discussed by the authors

- Firstly, Chou et al⁶² investigated a potential direct impact of HBV on RPE cells. In vitro, they induced an overexpression of HBx protein, the pathogenic X protein of HBV using ARPE-19 cells (a mainly immortalized human RPE cell line). Using ultraviolet (UV) and blue light exposures to induce oxidative stress and subsequent cellular damage, they showed that HBx-transfected cells had reduced cell viability and reduced clonogenic survival compared to mock-transfected cells. This suggests that HBV infection could sensitize RPE cells to UV or blue light-induced cell death. Furthermore, differentially expressed genes between HBx-transfected and mock-transfected cells are described both before and after UV exposure, indicating a downregulation of various DNA repair pathways by HBx. Nevertheless, although HBV biomarkers are frequently reported in tears, aqueous humor,^{64–67} cornea, or lenses,^{68,69} the presence of HBV within AMD-affected tissues remains unclear. If hepatitis B surface antigen was detected in the subretinal fluid of an HBV-positive patient,⁷⁰ to our knowledge, no study has investigated the presence of HBV in RPE cells or in retinal samples.

- Secondly, a more indirect mechanism has also been suggested: HBV-induced antibodies or effector lymphocytes

could cross-react with the retinal S-Ag, a major component of photoreceptor cells, which has sequence homology with the HBV DNA polymerase,⁷¹ and thus lead to subsequent inflammation and/or tissue damage (even in the absence of the virus within the eye). Although the existence of an autoimmune component in the development of AMD remains debated, elevated levels of retinal autoantibodies were described in patients with AMD compared to controls^{72,73} and, interestingly, higher rates and titers of autoantibodies against retinal S-Ag were also described among HBV healthy carriers compared to controls.⁷⁴

- Finally, HBV infection may also indirectly activate the alternative complement pathway, which is an important player in the development of AMD. Indeed, in vitro, HBx protein has been shown to downregulate the CFH (an inhibitor of the alternative complement pathway).⁷⁵

Hepatitis C Virus. In 2021, another study⁷⁶ based on the Taiwanese Longitudinal Health Insurance Database 2000 investigated the risk of incident AMD in 13 300 subjects over 18 years old with a diagnosis of chronic hepatitis C virus [HCV] infection and 26 600 propensity score-matched subjects without HCV. Subjects coinfecting with HBV or HIV were excluded. The propensity score included age, sex, socioeconomic characteristics, and some comorbidities. An increased risk of AMD was observed among HCV-infected subjects (aHR = 1.22 95%CI [1.09–1.35], $P < 0.001$) compared to controls. The association remained significant for nonexudative AMD (aHR = 1.22 95%CI [1.09–1.37], $P < 0.001$) but not for neovascular AMD (aHR = 1.17 95%CI [0.84–1.61], $P = 0.35$). Because interferon therapy is known to induce retinopathy,^{77,78} the incidence of AMD was compared between 1973 HCV-infected subjects treated by pegylated interferon and ribavirin and 3946 HCV-infected untreated subjects matched by a propensity score (including age, sex, socioeconomic factors, and some comorbidities). No statistically significant difference was found between being treated and AMD (aHR = 1.07 95%CI [0.81–1.43], $P = 0.24$) or its subtypes (neovascular AMD: aHR = 1.36 95%CI [0.58–3.22], $P = 0.497$; nonexudative AMD: aHR = 1.04 95%CI [0.77–1.41], $P = 0.78$). Based on these results, the authors conclude that the increased risk of AMD is probably linked to HCV infection itself and not to treatment by pegylated interferon and ribavirin. Certain limitations (previously mentioned for other studies carried out on this database) should be considered: a risk of underdiagnosis because of the absence of active screening for both pathologies, the lack of information on smoking or genetics, and a risk of follow-up bias increasing the probability of a diagnosis of AMD in subjects followed for HCV infection.

Three other studies investigating the role of HCV infection reported negative results.^{56,57,60} In a population of patients with AIDS included in the LSOCA cohort (2 studies by Jabs et al previously described in the HIV section), being coinfecting with HCV was not associated with an increased prevalence⁵⁶ or incidence⁵⁷ of intermediate-stage AMD. Using data from a South Korean cross-sectional study (previously described in the HBV section), Roh et al⁶⁰ also reported a lack of association

between HCV infection and AMD. However, this result suffers from low statistical power (only 1 HCV-infected subject with AMD).

Regarding the underlying pathophysiological mechanism, few elements have been discussed. It has been suggested that HCV could act directly within the eye. Indeed, HCV is known to infect certain compartments of the eye including the tears, the cornea, and the aqueous humor⁷⁹ and has also been associated with various ophthalmological disorders,⁷⁹ including retinal damage (retinal pigment epithelitis,⁸⁰ ischemic retinopathy⁷⁷). However, no information is available on the precise mechanism of action, which may involve the virus itself or the immune response to it.

Varicella-Zoster Virus. In 2019, using data from the Taiwanese Longitudinal Health Insurance Database 2005, Ho et al³⁰ analyzed the associations between the onset of herpes zoster ophthalmicus (HZO) and the incidence of neovascular AMD. Indeed, because of a reactivation of VZV, HZO can be associated with ocular complications which are mostly located in the anterior segment of the eye and, more rarely, in the posterior segment.⁸¹ Comparing 1148 subjects aged >40 with HZO and 5740 controls matched by propensity score (including age, sex, socioeconomic factors, and some comorbidities), the authors observed an increased risk of neovascular AMD among HZO subjects (aHR = 4.62 95%CI [2.59–8.24], $P < 0.001$). Of note, this study suffers from the same limitations as those previously described for other studies using this database.

Regarding the suspected underlying mechanisms, VZV can infect the retina as shown by cases of necrotizing retinopathy which occur in severely immunosuppressed patients with HZO.⁸² To our knowledge, there are no data on VZV infection in the retina outside the context of immunosuppression. The authors suggested that retinal VZV infection could lead to local chronic inflammation, vasculopathy,⁸³ as well as deposits of amyloid- β , which is a known component of drusen. Indeed, increased amyloid concentrations and aggregation were seen in vitro after VZV infection⁸⁴ and in vivo in the plasma of subjects with an acute clinically diagnosed zoster.⁸⁵

Cytomegalovirus. Because of its well-described role in promoting angiogenesis (reviewed in Caposio et al⁸⁶) and the existence of rare cases reporting ocular neovascularization during *Cytomegalovirus* (CMV) retinitis,⁸⁷ the potential involvement of CMV has been investigated in neovascular AMD.

An American cross-sectional study (previously described in the *C. pneumoniae* section)³⁶ compared the titers of plasma anti-CMV IgG between 47 subjects with neovascular AMD, 36 with nonexudative AMD, and 67 controls. Although no data on the proportion of CMV-positive subjects was presented, univariate analyses highlighted that subjects with neovascular AMD had higher levels of anti-CMV IgG than both controls ($P = 0.02$) and subjects with nonexudative AMD ($P = 0.06$). The authors hypothesize

that higher levels of anti-CMV IgG indicate a recent reactivation of the virus or a greater “total body burden of chronic CMV infection.” Conversely, there was no difference between subjects with nonexudative AMD and controls ($P = 0.83$). It should be noted that, although not statistically significant, the control group tended to be younger than the 2 AMD groups (71.6 ± 10.2 vs. 77.2 ± 7.39 in the nonexudative AMD group and 79 ± 6.49 in the neovascular AMD group; $P = 0.24$), suggesting a potential residual confounding bias.

Regarding the underlying mechanisms, several hypotheses have been made.

- Firstly, because monocytes are a major site of CMV latency, the virus can easily disseminate through the body and infiltrate various tissues. In the eye, CMV-infected monocytes become tissue-resident macrophages, cells which are suspected to have a direct role on the onset of AMD.^{88,89} Using C57BL/6 mice intraperitoneally infected with murine CMV, Cousins et al⁸⁹ highlighted larger laser-induced CNV lesions in infected mice compared to controls. Their results suggested that this was not due to direct infection of choroidal tissues by murine CMV but rather related to the activation of CMV-infected macrophages towards a proangiogenic M2 phenotype.

- Secondly, infected macrophages can help to disseminate the virus to other types of cells within the eye. Among various types of mice intraperitoneally infected with murine CMV shortly after birth (i.e., with a not completely mature immune system that may favor virus dissemination),^{90–92} an American team highlighted that murine CMV establishes latent infection within the choroid and in a few RPE cells^{90,91} and reactivates following systemic immunosuppression.⁹¹ Latent ocular infection was also associated with increased levels of inflammatory and angiogenic factors (including IL-6 and VEGF)^{90,91} as well as retinal and choroidal pathologies (including deposits at basal and apical aspects of the RPE, photoreceptor degeneration, and CNV).^{90–92} To note, the number of macrophages observed in CNV lesions were similar in infected and uninfected VEGF-A^{hyper} mice and did not represent the majority of VEGF-positive cells.⁹⁰ Subsequently, the authors also investigated the presence of CMV within human ocular tissues. Using droplet digital PCR assay performed on fresh eyes from 24 human cadavers (none of them having a known history of AMD), Xu et al highlighted that CMV DNA was detected in posterior eye cups (choroid/RPE) of 4 individuals (16.7%).⁹⁰ Conversely, CMV DNA was not found in any of the anterior segment samples and very low DNA copy numbers were detected in the neural retina of a fifth individual (4.2%). Although the precise type of cells infected remains to be determined, these results led them to suggest that the choroid/RPE could be a fairly common site of latency for CMV in humans.

- Finally, a nonspecific impact of CMV on AMD has also been proposed,³⁶ suggesting that infections by various pathogens (grouped under the concept of “total pathogen burden”) could shape the immune response and favor the onset of AMD. Indeed, a study by Zinkernagel et al,⁹³ using BALB/c mice intraperitoneally infected with murine

CMV, showed that systemic murine CMV infection also led to an increased number of activated microglial cells within the subretinal space (located between the photoreceptors and the RPE and normally devoid of microglia), indicating low-grade inflammation implicated in AMD.

Human Herpes Virus 6A. Fierz et al⁹⁴ published a hypothesis paper discussing the role of human herpes virus 6A (HHV-6A) in the onset of GA, mainly because of its known interaction with CD46, a membrane-bound inhibitor of complement activation. Indeed, serving as an HHV-6A receptor to enter cells, CD46 is downregulated following infection with HHV-6A. Thus, Fierz et al hypothesized that HHV-6A infection could explain the downregulation of CD46 seen in RPE cells at very early stages of GA⁹⁵ and its potential influence on disease progression as CD46 knockout mice have been shown to spontaneously develop nonexudative AMD.⁹⁶

Other Types of Studies. Four studies investigated the hypothesis of the involvement of infectious agents in the occurrence of AMD without focusing on a particular infectious agent.

- Using the American Framingham Heart and Eye Studies, an exploratory analysis published in 1977 evaluated potential risk factors for AMD⁹⁷ and highlighted an increased risk of developing a “senile macular degeneration” for participants with a history of lung infections (analyses stratified by age categories and sex; no details on the pathogen involved) (As this study precedes the publication of all current widely used classification systems of AMD, we have refrained from harmonizing the term “senile macular degeneration.” According to the authors “senile macular degeneration” was positive when diagnosed by the definitive examiner together with best corrected central visual acuity of 20/30 or worse in the same eye).

- More recently, in 2016, aiming to explore interactions between genetic and environmental factors, Hoh Kam et al⁹⁸ exposed CFH knock out mice to a pathogen free environment or to a conventional open environment during 9 months and investigated the effects on the retina. Compared with mice kept in a pathogen free environment, those in a conventional open environment presented (1) a higher number of macrophages in the outer retina and (2) higher levels of proinflammatory cytokines (TNF- α and IL-1 β) in the outer retina and of stress markers (glial fibrillary acidic protein and vimentin) in the inner retina. Consistently, they also presented a reduced number of photoreceptors in the outer nuclear layer. Moreover, the authors stated that the results obtained with these CFH knock out mice were not found with wild-type mice (data not shown in the article). The authors conclude that, in genetically predisposed immunocompromised mice, environmental factors may trigger chronic inflammation in the retina and subsequent loss of photoreceptors.

- In 2015, Brosig et al⁹⁹ stimulated cultured human RPE cells with analogs of (1) viral double-stranded RNA (dsRNA) (an intermediate of virus replication) and (2) viral/bacterial DNA to explore their effects on gene expression.

Viral dsRNA induced the expression of the angiogenic factor basic fibroblast growth factor (but not of VEGF), of some inflammatory cytokines (IL-1 β , IL-6, TNF α , monocyte chemoattractant protein-1, and macrophage inflammatory protein-2) and of complement proteins (complement factor B—the main activator of the alternative complement pathway—and to a lesser extent C5, C9, CFH but not C3). Conversely, viral/bacterial DNA had a lesser impact on gene expression, limited to an upregulation of some complement proteins (C5, C9). In view of these results, although the authors point out that differences in the concentrations used for viral RNA versus viral/bacterial DNA may partially explain the differences found, they concluded that alterations of RPE cells may be induced by viral dsRNA rather than viral/bacterial DNA.

- In 2021, a Chinese study investigated the presence of intraocular microbiota in the aqueous humor of patients undergoing cataract surgery with either AMD (n = 20), glaucoma (n = 26) or without other ocular disease (n = 41) using metagenomic sequencing.¹⁰⁰ They highlighted the existence of a low-biomass intraocular microbiota. Moreover, although the intraocular microbiota of all patients and controls had in common to be mainly composed of bacteria, the 3 groups had significantly different alpha-diversities ($P < 0.05$). They also reported specific microbial signatures characterizing the intraocular environment of patients with AMD and glaucoma, suggesting that bacterial translocation may be associated with each condition. Regarding the underlying mechanisms, the bacterial translocation and also in general the confirmation of bacteria in the eye could be linked with the concept of ocular dysbiosis, which might favor certain eye diseases. However, it remains unclear if aging and disease lead to translocation of certain microbiota or if ocular microbiota exists since birth and only its composition might change due to disease.

Brief Overview of the Literature on the Oral and Gut Microbiota

Oral Microbiota. Previously, 2 literature reviews^{24,27} investigated potential links between oral microbiota and AMD, with a particular focus on periodontal disease. Periodontal disease (and its more serious form called periodontitis) is an inflammatory disease triggered by several bacteria in dental plaque.

Briefly, 6 human studies with variable methodologies^{101–106} investigated potential associations between various symptoms related to periodontal disease (fewer teeth, alveolar bone loss, presence of periodontal pockets, clinical attachment level, gingival index, periodontitis, etc) and AMD. Although a small case-control study found no associations between these symptoms and AMD,¹⁰¹ 4 cross-sectional studies with larger sample sizes,^{102–105} highlighted a greater frequency of these symptoms among AMD cases compared to controls. Notably, in 2 of these studies,^{104,105} significant associations were restricted to a subgroup of participants <60 years of age. Moreover, one population-based cohort study based on a Health Insurance database¹⁰⁶ showed an increased risk to develop AMD among subjects with

prevalent periodontitis. Karesvuo et al¹⁰² also investigated the salivary presence of 6 pathogens (*Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis* (*P. gingivalis*), *Tannerella forsythia*, *Campylobacter rectus*, *Prevotella intermedia*, and *Treponema denticola*) in 54 self-reported AMD cases and 1697 controls but failed to find any difference in prevalence of bacterial DNA in the saliva between the 2 groups. More recently, a Canadian study⁵² (previously described in the paragraph on nasal microbiota) highlighted that 13 oral bacterial communities had an increased relative abundance in neovascular AMD cases (n = 13) compared to controls (n = 5), using 16s ribosomal RNA sequencing. Among these, the authors cited the *Rothia* genus (13-fold higher; $P = 3.63E-18$), the *Propionibacteriales* family (eightfold higher; $P = 6.74E-09$), the *Staphylococcus* species (sevenfold higher; $P = 6.96E-05$), and the *Corynebacteriaceae* genus (sevenfold higher; $P = 2.33E-05$). Only the *Fusobacterium* genus (10-fold lower; $P = 1.00E-10$) and the *Bacilli* class (threefold lower; $P = 0.007$) had a decreased relative abundance in cases compared to controls.

Regarding the underlying mechanisms, the authors suggested that, secondary to periodontal disease, local inflammation, and ulceration of the periodontal epithelium could facilitate the systemic spread of either periodontal pathogens, microbial toxins (such as LPS), proinflammatory molecules, or immune complexes to distant sites such as the eye,²⁴ favoring the onset of AMD.

Using in vivo and in vitro experiments, Arjunan et al investigated the potential impact of *P. gingivalis* (one of the major periodontopathogenic bacteria) on AMD. They highlighted that *P. gingivalis* can enter ARPE-19 cells and significantly reduce the expression of autophagy related genes.¹⁰⁷ Subsequently, using C57BL/6 mice orally infected with *P. gingivalis*¹⁰⁸ with and without a polybacterial suspension (referred as biofilm), they observed an increased size of laser-induced CNV lesions, a reduced retinal thickness and the presence of inflammatory drusen-like lesions in infected mice. Using quantitative PCR, they also confirmed the presence of *P. gingivalis* as well as an upregulated expression of angiogenic, proinflammatory, immunosuppressive, and oxidative stress genes and a downregulated expression of anti-inflammatory and anti-oxidative genes in the retina of infected mice, which, together, may promote the development of AMD.

Gut Microbiota. In recent years, several literature reviews have been published on the link between gut microbiota and AMD. Thus, the objective here is not to provide an exhaustive discussion of all original articles but, rather, to provide a brief overview and recent and relevant references to which the reader can refer.

We have identified 5 studies reporting original data on the link between gut microbiota and AMD in humans. Of all, the gut microbial profile was analyzed using DNA from fecal samples. However, it has to be noted that the information obtained from fecal samples does not represent the complete picture within the gut.¹⁰⁹ Still, the available studies have revealed that specific bacteria are differentially abundant in patients with advanced AMD compared with healthy age-matched controls.^{110–114}

- In 2017, using metagenomic sequencing, a Swiss study by Zinkernagel et al¹¹⁰ observed an increased *Firmicutes/Bacteroidetes* ratio in patients with neovascular AMD (n = 12), compared with healthy controls (n = 11). They also highlighted an enrichment in genera *Anaerotruncus*, *Oscillibacter* and in species *Ruminococcus torques* and *Eubacterium ventriosum* as well as a decreased abundance of species *Bacteroides eggerthii*.¹¹⁰

- In 2020, also using metagenomic sequencing, another study by the same Swiss study team (Zysset-Burri et al) showed that patients with neovascular AMD (n = 57) were enriched in class *Negativicutes* and less abundant in genus *Oscillibacter* and species *Bacteroides* than controls (n = 58).¹¹¹

- A recent review published in 2021 mentioned unpublished data describing an enhanced abundance of the genera *Prevotella*, *Holdemanella*, and *Desulfovibrio* but reduced abundance of the genera *Oscillospira*, *Blautia*, and *Dorea* in patients with advanced AMD (n = 85) compared with healthy control subjects (n = 49).¹¹²

- In 2023, using 16s RNA sequencing, a case-control study from China¹¹⁴ reported increased abundances of *Bacteroides* and *Eubacterium_siraeum_group* genera and decreased abundances of *Blautia* and *Anaerostipes* genera in patients with advanced AMD (n = 30) compared with healthy controls (n = 17).

- In 2023, another case-control study from China using metagenomic sequencing¹¹³ observed a decreased *Firmicutes/Bacteroidetes* ratio in patients with neovascular AMD (n = 30) compared to controls (n = 30). They also observed an enrichment in genera *Veillonella* and *Lactobacillus* in patients with neovascular AMD, whereas the genera *Faecalibacterium*, *Anaerostipes*, *Blautia*, and *Eggerthella* were reduced. Furthermore, no difference regarding eukaryotic viruses was detected between patients with neovascular AMD and controls, whereas 50 phages were enriched and 62 phages depleted in patients with AMD. In order to identify common characteristics of AMD-associated gut microbial signatures in cohorts of different geographical origin, the authors integrated the publicly available datasets of the Swiss studies of Zinkernagel et al¹¹⁰ and Zysset-Burri et al.¹¹¹ They highlighted that the compositions of the gut microbiome differed significantly between the Chinese and Swiss data (with approximately 75% bacterial species with different abundance). In the combined analyses, 3 bacterial species (*Ruminococcus callidus*, *Lactobacillus gasseri*, and an unknown species-level genome bin from the *Prevotellaceae* family) were concordantly enriched, and 6 bacterial species (*Cloacibacillus evryensis*, *Slackia piriformis*, as well as unknown species-level genome bins from the *Alistipes* and *Bilophila* genera and the *Bacteroidaceae* and *Acidaminococcaceae* family) depleted in AMD. Furthermore, the analyses showed that a total of 12 phages were enriched in AMD and 41 phages were reduced.

All in all, if a distinct microbial signature linked to AMD exists, the reported discrepancies highlighted in these studies make it difficult to link specific shifts in microbial composition to AMD.^{25,115–117}

Several studies (in humans, animals, or in vitro) are also exploring the mechanisms potentially involved. Readers can refer in particular to the recent reviews by Zysset-Burri et al and Xiao et al^{25,111} for a more comprehensive discussion. In a nutshell, gut dysbiosis may impair the integrity of intestinal barrier allowing pathogens, bacterial endotoxins like LPS,^{118–121} cytokines (among others IL-6, IL-1 β , TNF- α , and VEGF-A), and other proinflammatory substances to pass into circulation, resulting in systemic inflammation, potential impairment of the immune response, and/or local ocular inflammation. Especially among subjects carrying the risk variant of the CFH gene, this could favor the development or progression of AMD.¹¹¹ Both reviews also discuss the impact of diet (in particular high glycemic and high fat diets) on gut microbiota suggesting that gut microbiota may be part of the associations found between diet and AMD. Some authors also suggested that the impact of gut dysbiosis on AMD might be mediated by changes related to their metabolic functions or to the bioavailability of some nutrients (e.g., zinc or carotenoid).²⁶

Discussion

This systematic literature review gives an overview of studies investigating a potential link between infectious agents and AMD. It highlights that, although an overall low number of studies have been published on the subject so far (only 40 original articles excluding studies focusing on oral and gut microbiota), this research topic has been of growing interest in recent years. Of the 40 studies examined in this review, only 1 predates 2000, 12 were published between 2000 and 2010 (mainly on *C. pneumoniae*), followed by a substantial increase to 27 studies post 2010 (to which we can add the majority of studies on oral and gut microbiota).

Regarding the type of pathogens involved, a wide range of infectious agents has been investigated, including various microbiota (nasal, pharyngeal, oral, and gut microbiota), 8 bacteria (*C. pneumoniae*, *C. trachomatis*, *C. psittaci*, *E. coli*, *H. pylori*, *M. pneumoniae*, *M. chelonae*, and *B. megaterium*), 6 viral species (HIV, HBV, HCV, VZV, CMV, and HHV-6A), and 1 yeast (*C. albicans*). Among them, most have been investigated anecdotally (only 1 or 2 related studies) or only to test the specificity of the association for another pathogen (e.g., infections with different types of *Chlamydia*). Only *C. pneumoniae*, CMV, and HBV received more attention, with 17, 6, and 4 studies, respectively. It should be noted that, although 4 studies exist for HCV, HCV was not the main focus of 3 of them.

The numerous pathophysiological mechanisms investigated underline the diversity of interactions between infectious agents and AMD players (Fig 2):

- On the one hand, an indirect role of infectious agents in the onset of AMD has been suggested (i.e., a role of infections located distant from the eye), mainly related to their interactions with the immune system. Some authors proposed that chronic exposure to pathogens (or to their products such as bacterial endotoxin or soluble fungal

ligands) could prime or partly activate immune cells located at a distance from the eye, leading to proinflammatory signaling and activating further inflammatory pathways or effector functions.¹²² Subsequently, such chronic systemic inflammation and/or the recruitment of immune cells within the eye could favor the onset of AMD. Thus, this hypothesis does not imply a particular pathogen but, rather, suggests an unspecific role of various infectious agents using a common mechanism (e.g., gut microbiota, oral microbiota, nasal or pharyngeal microbiota, *C. pneumoniae*, HIV, etc). In parallel, mechanisms more specific to a particular infectious agent (again at a distance from the eye) have also been proposed, including cross-reactivity phenomena because of sequence homologies between retinal and infectious proteins (e.g., HBV DNA polymerase and retinal S-Ag) leading to retinal damage.

- On the other hand, a direct role of infectious agents has been discussed implying potential infection of various cells types within AMD-related tissues (e.g., RPE cells, endothelial cells, macrophages, retinal microglia). Although only a few studies have investigated the presence of pathogens within AMD-related tissues in humans (4 studies on *C. pneumoniae*, 1 study on CMV, and 1 on *B. megaterium*), several mechanistic avenues have been considered in experimental studies. The ability of *C. pneumoniae* and CMV to infect monocytes, their potential secondary dissemination to AMD-related tissues during macrophage recruitment, and the potential to locally infect other cell types (e.g., endothelial cells for which *C. pneumoniae* has a particular tropism, RPE cells, which are the first line of defense against external pathogens, etc) have been most extensively studied. Some authors also suggest that the presence of dysbiosis in various microbiota (intestinal, oral, etc) could favor the translocation of pathogens from the bloodstream to ocular structures. Presence of infectious agents within AMD-related tissues have thus been associated with a proinflammatory and proangiogenic environment, which could favor AMD onset, in particular neovascular AMD. A role for potential infection of RPE cells by HHV-6A or HBV has also been suggested at other AMD stages like GA. Moreover, numerous other mechanisms implicated in AMD could also suggest an involvement of infectious agents: oxidative stress and the inflammatory component and in particular the activation of the alternative complement pathway whose main trigger is microbial in nature but also mechanisms such as mitochondrial and autophagy dysfunctions,¹²³ accumulation within drusen^{1,124} of lipids,¹²⁵ amyloid peptides,¹²⁶ zinc¹²⁷ and iron,¹²⁸ which could be implicated in antimicrobial defense.

- Finally, certain risk or protective factors associated with AMD¹ could also have a link with infections: (1) advancing age leading to the progressive onset of an immunosenescence and subsequent poorer infection control, (2) exposure to light which is a risk factor for certain infections (e.g. reactivation of certain herpes viruses),¹²⁹ and (3) carotenoid and zinc supplementation, which could have an impact on antimicrobial defense.^{127,130,131}

Despite these numerous hypotheses, the level of evidence remains low even for the 3 most studied pathogens:

- Briefly, results from human studies focused on *C. pneumoniae* are mixed, with 10 human studies in favor and 7 against its implication in AMD. Most of the negative studies were based on serological data, some of which suffering from methodological weaknesses (low numbers, cross-sectional or case-control design, little consideration of confounding, or interaction factors). To note, among the best quality studies, the one performed within the prospective “Cardiovascular Health and Age-related Maculopathy Study” presents a striking result suggesting a major increase in the risk of progression of early AMD in subjects with both high levels of anti *C. pneumoniae* IgG and the CC risk genotype of the CFH gene, a result that would need to be replicated. Two out of 3 studies also demonstrated the presence of *C. pneumoniae* within CNV lesions, suggesting a potential direct effect of the bacteria on AMD-related tissues. In vitro and animal studies also highlighted a proinflammatory and proangiogenic effect of *C. pneumoniae* infection, which could favor the onset of CNV.

- Regarding studies on HBV, an increased risk of AMD in infected subjects was demonstrated in 3 independent databases (2 South Korean cross-sectional studies and the Taiwanese Longitudinal Health Insurance Database 2000 which was the subject of 2 articles). The replicability of these results is intriguing and warrants further investigation of this hypothesis, particularly in countries with high endemicity of HBV infection.¹³² Furthermore, several underlying mechanisms have been suggested either through a cross-reactivity phenomenon due to sequence homology between retinal and viral components or through a sensitization of HBV-infected RPE cells to UV or blue light-induced cell death through downregulation of DNA repair pathways (although, to our knowledge, evidence of RPE cell infection by HBV in humans remains to be established).

- Regarding CMV, one postmortem study highlighted that CMV DNA was detected in posterior eye cups (choroid/RPE) of 16.7% of human cadavers, suggesting that the choroid/RPE could be a fairly common site of latency for CMV. Nevertheless, its impact on AMD remains poorly studied in humans with only 1 small cross-sectional study highlighting an association between high anti CMV IgG levels and neovascular AMD. Experimental studies are more numerous and suggest an impact of CMV on AMD onset through proangiogenic and proinflammatory mechanisms.

Our scoping review has some limitations. Given the diversity of terms associated with infectious agents, we had to limit our review to a single electronic database (PubMed) and search for terms only in titles and abstracts to ensure the feasibility of our work. Consequently, as mentioned above, we may have missed some articles studying the impact of infectious agents on the eye but without a specific focus on AMD (and therefore without AMD-related terms in the title and/or abstract). This was particularly the case for current studies focusing on SARS-COV2, in which SARS-COV2 infection related changes

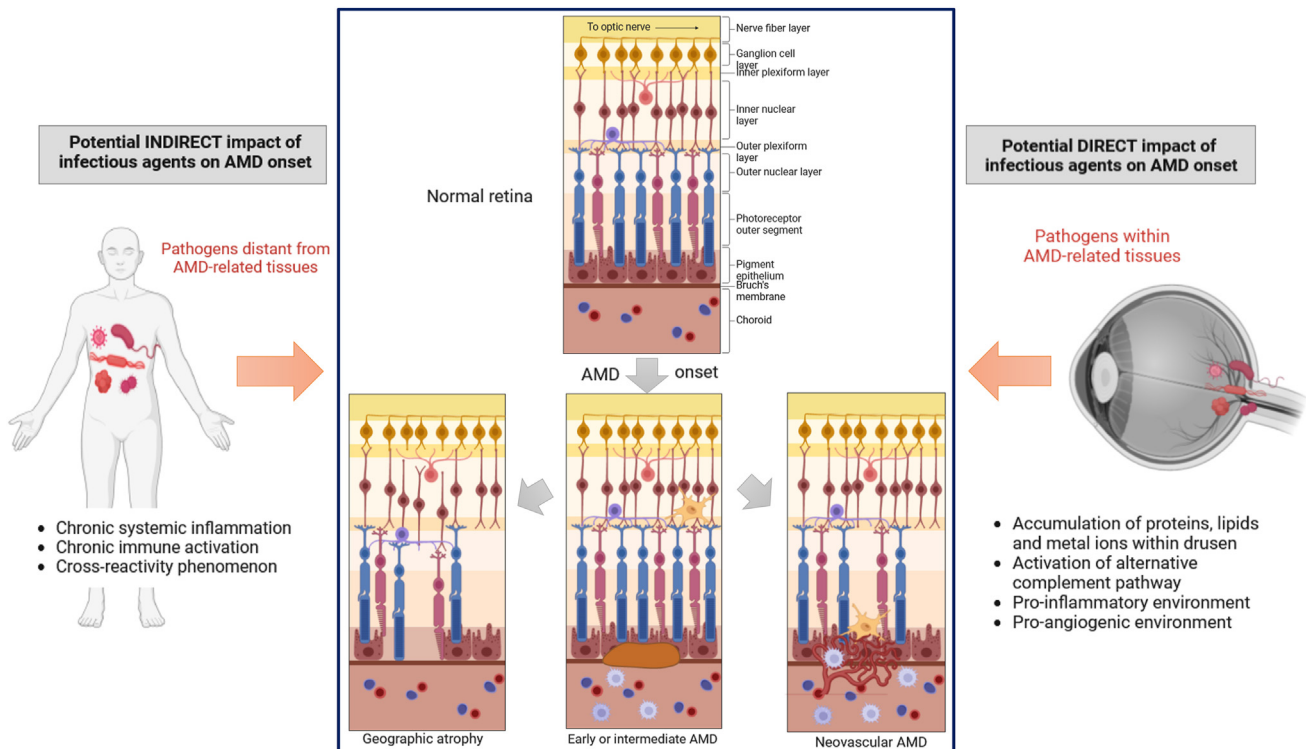


Figure 2. Stages of age-related macular degeneration (AMD) and potential involvement of infectious agents. Age-related macular degeneration is clinically classified into 3 stages according to the severity of fundus lesions. Early and intermediate AMD are described by the onset of drusen underneath the RPE and accumulation of immune cells (subretinal microglia and choroidal macrophages). Advanced AMD includes geographic atrophy, retinal thinning due to loss of photoreceptors, RPE cells, and choroidal capillaries, and neovascular AMD, invasion of new abnormal choroidal blood vessels and accompanying macrophages into the subretinal and/or sub-RPE space. Both geographic atrophy and neovascular AMD can also coexist in 1 eye (which is not shown in this figure). Several studies suggest a potential involvement of infectious agents in the development of AMD-related lesions: An indirect role of infectious agents has been suggested (i.e., a role of infections located distant from the eye), mainly related to their interactions with the immune system. Also, a direct role of some infectious agents has been discussed implying potential infection of various cell types within AMD-related tissues (e.g., RPE cells, endothelial cells, macrophages, retinal microglia). RPE = retinal pigment epithelium. Figure created with [BioRender.com](https://www.biorender.com).

were reported on ocular structures including the retina. However, the context in which existing studies were set up is a limiting factor for investigating the impact of SARS-COV2 on the development of AMD (short follow-up time, scarcity of uninfected controls, absence of information on the presence/severity of AMD lesions before infection, etc). Furthermore, our search algorithm was not designed to identify studies investigating only certain components of pathogens (such as certain endotoxins, for example). Finally, our review was limited to articles in English and, as a result, 2 articles in Russian dealing with our research theme could not be included.

In conclusion, the currently available data do not clearly speak in favor or against the implication of infectious agents in AMD. Future studies combining human, animal and in vitro experiments for the same pathogen are needed to improve our understanding. Experimental studies will be decisive to decipher potential underlying mechanisms and

could help to guide the choice of AMD stage to investigate for each suspected pathogen. Studying the interactions between an aging immune system and infectious agents also seems essential (i.e., could the onset of immunosenescence with advancing age explain a certain latency between the time of infection and the appearance of lesions?). Further anatomopathological studies assessing the presence or absence of pathogens within human AMD-related tissues will also help clarify the plausibility of a direct role for infectious agents. Large-scale epidemiological studies are needed to better take into account confusion bias and potential gene-environment interactions (i.e., is infection by a pathogen associated with the occurrence of AMD lesions only in genetically predisposed subjects?). Overall, an intensification of research efforts on the infectious hypothesis and AMD seems essential, given the potential repercussions in terms of diagnosis, prevention, and treatment.

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Abbreviations and Acronyms:

aHR = adjusted hazard ratio; **AMD** = age-related macular degeneration; **aOR** = adjusted odds ratio; **B. megaterium** = *Bacillus megaterium*; **C. albicans** = *Candida albicans*; **CFH** = complement factor H; **CMV** = Cytomegalovirus; **CNV** = choroidal neovascularization; **C. pneumoniae** = *Chlamydia pneumoniae*; **C. psittaci** = *Chlamydia psittaci*; **C. trachomatis** = *Chlamydia trachomatis*; **dsRNA** = double-stranded RNA; **E. coli** = *Escherichia coli*; **GA** = geographic atrophy; **H. pylori** = *Helicobacter pylori*; **HBV** = hepatitis B virus; **HCV** = hepatitis C virus; **HHV-6A** = human herpes virus 6A; **HIV** = human immunodeficiency virus; **HZO** = herpes zoster ophthalmicus; **IgG** = immunoglobulin G; **IHC** = immunohistochemistry; **IL** = interleukin; **LPS** = lipopolysaccharide; **LSOCA** = Longitudinal Study of the Ocular Complications of AIDS; **M. chelonae** = *Mycobacterium chelonae*; **MESA** = Multi-Ethnic Study of Atherosclerosis; **M. pneumoniae** = *Mycoplasma pneumoniae*; **OCTA** = OCT angiography; **PCR** = polymerase chain reaction; **P. gingivalis** = *Porphyromonas gingivalis*; **RPE** = retinal pigment epithelium; **SARS-COV2** = severe acute respiratory syndrome coronavirus type 2; **TNF** = tumor necrosis factor; **UV** = ultraviolet; **VZV** = varicella-zoster virus.

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